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LI. A NOTE ON THE REDUCTION POTENTIAL OF CYSTEINE-CYSTINE MIXTURES.

By JNANENDRA CHANDRA GHOSH
AND SAILESH CHANDRA GANGULI.

From the Department of Chemistry, University of Dacca.

(*Received December 11th, 1933.*)

Ghosh *et al.* [1932] have shown that in solution of $p_H > 7$, the electrolytic reduction of cystine to cysteine at a mercury electrode sets up a reversible potential system at the electrode. The potential of the system was determined by the concentration of the oxidant and the reductant and agreed with the thermodynamic formula

$$-E_h = E_0 - \frac{RT}{F} p_H - \frac{RT}{F} \log \frac{[\text{Cysteine}]}{\sqrt{[\text{Cystine}]}} \qquad \ldots\ldots(1),$$

where $E_0 = +\cdot 079$.

More recently Green [1933] has shown that the evidence brought forward by Ghosh *et al.* could be substantiated if the potential were measured at the particular mercury surface at which the cystine was reduced. But if the potential were measured at a new mercury surface, introduced into the system by an auxiliary electrode, a difference of about 60 mv. was at once observed and the potential at the auxiliary mercury electrode conformed to Dixon and Quastel's [1923] equation

$$E = E_0 - \frac{RT}{F} p_H - \frac{RT}{F} \log [\text{Cysteine}] \qquad \ldots\ldots(2).$$

Quoting Barron *et al.* [1929] on the possibility of complex formation between cysteine and mercury in absence of oxygen when a galvanic cell is set up, Green confirmed the observation of Ghosh *et al.* that after electroreduction of cystine, H_2S did not give a precipitate, but he did not agree with their conclusion that no complex formation occurred in their experiments, and suggested that such a complex was indeed formed but was confined to the mercury surface.

It is however well known that a mercury surface when exposed to air is covered with an adsorption layer. Reeser and Sissing [1922] found the thickness of the adsorbed layer to be between 22 and 34 Å. McBain [1932] believes this layer to be a film of mercuric oxide, as it is not affected by evacuation for eight hours at room temperature using a Gaede pump.

The present authors in their previous work were guided by the suspicion that a film of adsorbed oxygen or oxide on the surface of the mercury electrode was responsible for the results of Dixon and of Barron *et al.* This film could not be removed by bubbling an inert gas like nitrogen, but could only be destroyed by a cathodic reduction process at the surface. The experimental method of Green with slight modifications is well suited to test directly the accuracy of this hypothesis.

Chemically pure N_2 gas prepared from NH_4Cl and $NaNO_2$ was dispersed into minute bubbles by means of a Jena gas filter and passed up a tower containing about 500 cm. length of a solution of alkaline pyrogallol prepared according to

the method of Haldane and Makgill [1933]. The complete absorption of all traces of oxygen was confirmed by passing the gas over a piece of white phosphorus which did not glow, showing that the oxygen pressure was less than 0·0007 mm.

The cell consisted of two chambers A and B connected by a stopcock T with 8 mm. bore. The inner portion of each chamber consisted of two tubes one within the other. The outer tube served as the nitrogen inlet and the inner tube as the siphon for contact with the calomel electrode. The outer limb of the siphon was filled with agar saturated with KCl. A platinum wire fused into the bottom of the vessel made electrical contact with the mercury electrode.

The chamber A was first filled with a buffer solution having the same p_H as the cystine solution. The bore of the stopcock T was also filled with buffer solution. The stopcock was then closed and the chamber B filled up with the buffered cystine solution. The apparatus was then set up as depicted in Fig. 1

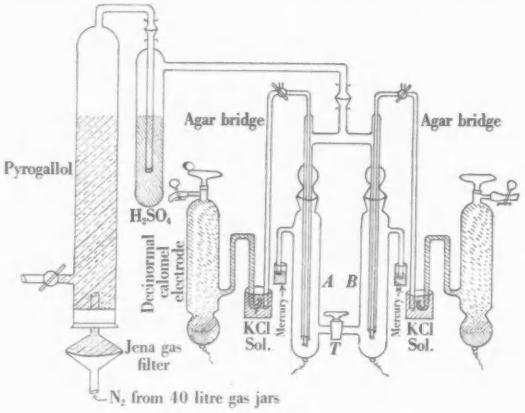

Fig. 1.

and the chambers de-aerated by a vigorous current of nitrogen. A current of 5 milliamperes was passed for 5–10 minutes between the mercury in the chamber B as cathode and a platinum anode in the KCl solution beyond the agar bridge. The mercury in the chamber A was similarly subjected to cathodic depolarisation for 3–4 hours. It is essential for the success of the experiment that the potential drop at the cathode in chamber A shall be sufficient to discharge hydrogen gas but much less than is necessary to discharge the alkali metal ions which were used to prepare the buffer solutions. The results become erratic if alkali metal amalgams are formed at the surface. Hence cathodic depolarisation for sufficiently long periods with very low currents is necessary. The stopcock T was now opened; by variation of the height of the mercury columns at the nitrogen outlets from the vessel as well as by passing a vigorous current of nitrogen for 3–4 hours, the solutions in both the chambers were well mixed. The potentials at the mercury surfaces in each chamber were then measured against a decinormal calomel electrode.

The results are given in Table I.

The potentials of the mercury surfaces in chambers A and B become ultimately the same and are given by the equation (I).

Table I.

p_H	Conc. cystine	Conc. cysteine	E_h in mv. Chamber *A*	E_h in mv. Chamber *B*	E_0 calc. according to (1)
7·00	0·00171 *M*	0·00099 *M*	−232	−230	0·082
7·50	—	—	−243	−242	—
8·00	—	—	−307	−308	—
8·50	—	—	−384	−383	—

The experimental results recorded above therefore confirm the hypothesis that an oxygen or oxide film on the mercury surface is responsible for setting up a potential in cystine-cysteine mixtures which follows the equation (2). Elimination of this film and complete removal of oxygen from the solution give rise to potentials which follow the thermodynamic equation (1).

It is well known that in presence of oxygen, complex formation of mercury with —SH compounds takes place rapidly. It is also probable that when a very limited supply of oxygen is available, as is the case in an adsorbed film of oxygen or oxide, mercury forms complexes with —SH compounds in preference to —S—S— compounds. The system Hg—HgS—H_2S gives potentials [Immerwahr, 1901] which would obviously depend only on the concentration of H_2S. The authors are of opinion that the Dixon potentials may be due to a similar system, where HgS has been replaced by a compound formed by interaction of the —SH compound with the oxide film at the mercury surface and H_2S replaced by sulphydryl compounds.

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LII. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

V. THE LYTIC EFFECT OF MIXTURES OF TWO LYSINS.

By ERIC PONDER.

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(*Received December 23rd, 1933.*)

THE only existing paper on this subject is one by Sen and Mitra [1929], in which a study of the lytic effects of mixtures of saponin, sodium taurocholate, sodium glycocholate and sodium oleate, in various proportions, led to the conclusion that the effects of two lysins acting together are probably additive in most cases, although in some instances the effect of the mixture may be greater than the sum of the individual effects. Sen and Mitra's experiments, however, are hardly conclusive, for two reasons. (1) No account is taken of the p_H changes which occur when two lysins such as saponin and sodium taurocholate are mixed together, which may result in either an acceleration or an inhibition of the action of one or both lysins. Sen and Mitra recognise this themselves. (2) The existing data are not presented in such a way as to be susceptible of exact analysis, and the conclusion that the effects of two lysins are additive in most cases really rests on nothing more substantial than an impression.

This paper is concerned with a re-investigation of the problem, and, as will be seen, leads to conclusions somewhat more definite than those of Sen and Mitra.

1. *Methods.*

Except in the case of systems containing sodium oleate, all the lysins were dissolved in an isotonic phosphate buffer at p_H 6·77, this buffer being selected because at this p_H the time-dilution curves for sodium taurocholate and sodium glycocholate are of the usual regular form and devoid of the "zones" so characteristic of these lysins in systems of certain p_H. In the case of systems containing sodium oleate the buffer used was a phosphate buffer at p_H 8·5, for this lysin is not sufficiently active in buffers of lower p_H. The cell suspension was a "standard" suspension consisting of the thrice washed cells of human blood finally suspended in 20 ml. of 0·9 % NaCl for every ml. of blood used. It is well to let the lysins stand for some hours before they are used, for as Sen and Mitra remark, their lytic activity often decreases with time.

To illustrate the procedure, consider the case in which the combined effects of saponin and sodium taurocholate are to be investigated quantitatively. First we plot a time-dilution curve at 25° for the saponin, the systems consisting of 0·8 ml. of various dilutions of the lysin (1 in 10,000, 1 in 20,000, *etc.*), 0·8 ml. of buffer, and 0·4 ml. of the standard suspension. Let the quantity of lysin present in each system be c_1 and the time for complete lysis t_1. Next we take a system consisting of 0·8 ml. of taurocholate, 1 in 2000 (say), 0·8 ml. of buffer and 0·4 ml. of suspension and find the time taken by it to show complete lysis. Suppose that it is the

same time as that taken by a dilution of 1 in 60,000 saponin (33·3γ in the system of total volume 2 ml.). Then 1 in 2000 taurocholate is equivalent to 1 in 60,000 saponin, or 1000γ of taurocholate to 33·3γ of saponin, equivalence being judged by the taking of equal times to produce complete lysis. Call this equivalent quantity of saponin c_3.

Now prepare systems containing both lysins. Each contains 0·8 ml. of the 1 in 2000 taurocholate, 0·8 ml. of various dilutions of saponin, and 0·4 ml. of cell suspension. Let the time for complete lysis be t_2. Referring to the time-dilution curve for saponin acting alone, we find t_2 corresponding to a quantity c_2; then, if the effects are additive, one would expect to find

$$c_3+c_1=c_2,$$

c_1 being the initial quantity of saponin present in the system containing the mixed lysins which haemolyses in time t_2, c_2 the quantity of saponin which, acting alone, would produce lysis in time t_2, and c_3 having the meaning defined above. I say that one would expect to find this relation, because it seems to follow so obviously from the reasoning, but, as a matter of fact, I shall show that the relation would be true under special conditions only, and that these are far from obvious.

If (c_3+c_1) be not equal to c_2, the activity of the mixture appears to be depressed below, or increased above, the sum of the activities of the two lysins by the factor $c_2/(c_3+c_1)$, and the value of this ratio can be used to measure the extent of the departure from a simple additive effect.

2. *Results.*

1. *Saponin and digitonin.* These lysins are both glucosides and have haemolytic properties which are similar in almost every respect. When mixed, however, their combined effect is much less than their individual effects added together, as can be seen from the values for the ratio $c_2/(c_3+c_1)$ which are given in Table I.

Table I.

Saponin γ	Digitonin γ 40	25	18
100	0·89	0·53	0·59
50	0·90	0·52	0·55
40	0·92	0·56	0·59
25	0·87	0·60	0·54
20	0·83	0·57	0·51

In the case of these two lysins, the inhibition is greater when the lysins are present in the smaller concentrations, but, as will be seen below, this is not an invariable rule. I may remark that the second decimal of the value given for the ratio is probably not very reliable.

2. *Saponin and sodium taurocholate.* These two lysins differ quite considerably in their haemolytic properties, but as in the case of saponin and digitonin, there is mutual inhibition.

Table II.

Saponin γ	Taurocholate γ 2000	1000	667
100	0·73	0·98	0·98
67	0·71	1·00	0·97
40	0·72	0·89	0·93
25	0·84	0·85	0·86
20	0·89	0·88	0·80

As in Table I there is a tendency for the inhibition to increase as the quantity of saponin becomes less, but it does not change in a similar way as the quantity of taurocholate is varied. The greatest inhibition of all, indeed, is met with when the bile salt is present in the greatest concentration.

3. *Saponin and sodium oleate.* These two lysins are quite unlike in many respects, and the activity of a mixture of the two is far greater than that which would result from summing the individual activities.

Table III.

Saponin γ	Oleate γ 200	100	67
100	1·74	1·63	1·41
67	1·52	1·67	1·40
50	1·39	1·60	1·37
40	1·34	1·50	1·26
33	1·16	1·37	1·05
25	1·09	1·11	1·03

The acceleration in this case is greatest when the lysin concentration is greatest and falls off as either the quantity of saponin or of oleate present in the system is reduced.

4. *Sodium taurocholate and sodium glycocholate.* In the case of these two similar haemolysins, we again find more than an additive effect, and again the acceleration is greatest when the lysins are present in greatest quantity.

Table IV.

Taurocholate γ	Glycocholate γ 1000	800	670
1330	2·14	1·44	1·27
1000	1·81	1·36	1·20
800	1·39	1·33	1·16
667	1·33	1·29	1·11
500	1·20	1·18	1·07
400	1·12	1·09	1·04

5. *Sodium taurocholate and sodium oleate.* This combination of lysins gives the most curious results of all:

Table V.

Taurocholate γ	Oleate γ 200	100	67
2000	0·62	0·78	0·76
1000	1·02	0·70	0·79
500	1·54	1·07	0·89
400	1·70	1·20	0·93

Here we find more than an additive effect, or less than an additive effect, depending on the concentrations of the haemolysins present in the system.

Discussion.

At first sight these results appear to dispose of Sen and Mitra's contention that the total effect of mixtures of lysins is "in most cases probably additive," for the ratio $c_2/(c_3+c_1)$ is generally greater or less than unity. Before drawing such a

conclusion, however, we must carefully scrutinise the principles on which the investigation is based.

To simplify matters, let us first assume that both lysins, which we shall refer to as A and B, act on the same cell component. Existing theory states that lysis is complete when $x\gamma$ of lysin are used up in combining with a proportional quantity of cell component. Suppose that this occurs when lysin A transforms the component, and when $x=25$. Further, suppose that complete lysis takes time t_1 when A is used. When B is used, let the time be t_1 also: then we would say that the quantity c_2 of B has the same activity as the quantity c_1 of A, for both give complete lysis in the same time t. If the two lysins were acting together in these same concentrations, we should at first sight expect the time taken for complete lysis to be the same as that taken by (c_1+c_1) of A, if A were acting alone.

But a little consideration will show that this is only so if the value of n in the expression for the fundamental reaction for A and B is the same, *i.e.* the kinetics of the reaction between the cell component and A and between the cell component and B must both be described by the expression

$$kt=\frac{p}{p-1}\left\{c^{\frac{p-1}{p}}-(c-x)^{\frac{p-1}{p}}\right\},$$

with the same value of $p=1/n$. Or, in words, if two lysins produce complete haemolysis by transforming the same amount of cell component in the same time, they will transform different amounts of cell component in shorter times, unless the value of n is the same for the time-dilution curves of both lysins. This is a very important point, and is related to another point which has been discussed elsewhere [Ponder, 1932], *viz.* that if we have a quantity of lysin c_1 which, as the result of the presence of some inhibitor, appears to have been reduced to the smaller quantity c_2, then the quantity of lysin rendered inert by the inhibitor is (c_1-c_2) only if the value of n is the same in the presence and in the absence of the inhibitor.

Let us take a concrete example, in which 100γ of lysin A (*e.g.* saponin) act on cells to produce complete lysis when $x=25$. Suppose that $n=1{\cdot}0$ and $1/k=10$. The relation between t and the quantity of component transformed is then shown in Fig. 1, curve A, and the time for complete lysis is 1·23 minutes. Now let 100γ of B act, and suppose that $n=2{\cdot}0$ and $1/k=0{\cdot}59\times10^{-3}$. Curve B of the same figure shows the relation between t and x, and again complete lysis occurs in 1·23 minutes. In these concentrations the two lysins are accordingly equal to each other, at least when the time for complete lysis is used as a criterion. But if the transformation of $12{\cdot}5\gamma$ is taken as a criterion, they are unequal, for A transforms this quantity in 0·57 minute and B in 0·51 minute. And similarly for the transformation of any quantity less than 25γ.

Now suppose that we are ignorant of this effect of the different values of n, and mix 100γ of A with 100γ of B. Then we again get complete lysis when 25γ of lysin are transformed, and referring to the A-curve (arbitrarily used as a standard) we expect this to occur in 0·57 minute, for then if n were 1·0 for both A and B, we should get a transformation of $12{\cdot}5\gamma$ of A and $12{\cdot}5\gamma$ of B. In fact, we get 25γ used up in combining with the cell component in 0·54 minute as the result of 12γ of A being used up, and 13γ of B. This looks like an acceleration, and referring to the time-dilution curve for A, we find $c=200\gamma$ giving complete lysis in 0·57 minute and $c=210\gamma$ giving complete lysis in 0·54 minute. In such a case we should find the ratio $c_2/(c_3+c_1)$ to be 1·05, although in fact the effects of the two lysins were strictly additive.

This apparent acceleration is not very spectacular, but it becomes far greater if we use a smaller quantity of lysin, *e.g.* 35γ of A and $42{\cdot}5\gamma$ of B[1]. These both produce lysis in 5·44 minutes if the values of n and of $1/k$ are the same as the above. If n were the same for A and B, and the values of $1/k$ so adjusted as to give $t=5{\cdot}44$ for $x=25$, then we should have 25γ transformed in 1·9 minutes in a system containing both A and B in the above concentrations. In fact, when $n=2{\cdot}0$ for B and 1·0 for A, we get complete lysis in 1·25 minutes, for at this value

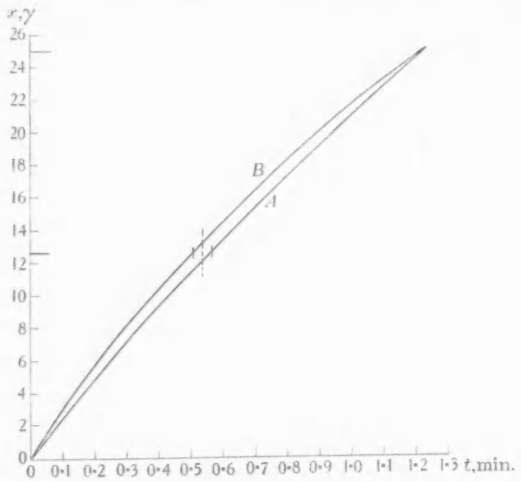

Fig. 1. Two curves showing the relation between time and the quantity of lysin transformed, A, when $n=1{\cdot}0$, B, when $n=2{\cdot}0$. For details, see text.

of t we have $8{\cdot}25\gamma$ of A transformed and $16{\cdot}75\gamma$ of B, the total being 25γ. Referring to the time-dilution curve for A, we get $t=1{\cdot}25$ when $c=99\gamma$ and $t=1{\cdot}9$ when $c=70\gamma$. So the ratio in this case turns out to be 1·4, which represents quite a considerable acceleration, although the activities of the two lysins are still strictly additive.

By the same reasoning, if we select as the standard curve that for the lysin with the higher value of n, instead of that for the lysin with the lower value of n, as above, the apparent accelerations become replaced by apparent inhibitions of the same magnitude.

It would be possible, at the expense of a rather unwarrantable amount of trouble, to investigate each one of the ratios shown in the preceding tables and to find out if the observed departures of the $c_2/(c_3+c_1)$ ratios from unity are such as could be accounted for by one of these "pseudo-accelerations" or "pseudo-inhibitions," but a conclusion may be arrived at in a quicker way, for under all circumstances the pseudo-acceleration or pseudo-inhibition is more obvious in the systems containing the more dilute lysins. We can accordingly draw up a

[1] If two concentrations of two lysins produce complete lysis in the same time, the same fractions of these concentrations produce lysis in different times, unless n is the same for both. This is so obvious that it requires no discussion.

Table showing what we should expect to find if the departures of the ratio from unity were the result of such pseudo-accelerations and inhibitions, and also what we find in fact.

Table VI.

Standard lysin	n	Second lysin	n	Result predicted	Result found	Increase or decrease in dilute system
Saponin	1·8	Digitonin	2·5	Accel.	Inhib.	Increase
Saponin	1·8	Taurocholate	1·6	Inhib.	Inhib.	Increase
Saponin	1·8	Oleate	1·3	Inhib.	Accel.	Decrease
Taurocholate	1·6	Glycocholate	1·2	Inhib.	Accel.	Decrease
Taurocholate	1·6	Oleate	1·3	Inhib.	Both accel. & inhib.	

It is therefore quite clear that even if all allowance is made for the occurrence of these pseudo-accelerations and inhibitions, we have no evidence that the activities of these haemolysins are in fact additive. Indeed, if we were to go through the labour of making the necessary corrections, the departure of the ratio from unity would be increased in as many cases as it would be decreased.

We are thus forced to one of two conclusions: either (*a*) that the effects of two lysins are not additive, or (*b*) that the additive effect is obscured by the lysins reacting with one another in such a way as mutually to inhibit or enhance each other's activities. This they might do by reacting with each other in solution, as two colloids very frequently do, or by interfering with each other in the region of the red cell surface. The two possibilities (*a*) and (*b*) are not mutually exclusive, but, as I shall show, the second is far the more likely.

Let us see what the hypothesis that the activities of two lysins are not additive amounts to, the term additive being used in the sense of this paper, and all inhibitory and acceleratory effects of the lysins on each other being either excluded or allowed for. Under such circumstances, the effects of the lysins must be additive if they affect the same cell component. If they affect two different cell components, their effects will not be additive unless we imagine that the result of partial destruction of two different and spatially separated components is equal, in an additive sense, to the much greater destruction of a single component. There is no precedent for such an idea, which is highly improbable in itself: in fact, if the two lysins attack different cell components, their effects will almost certainly not be additive. The hypothesis that the activities are not additive is therefore tantamount to a hypothesis that the lysins react with different components and demands that they shall not react with the same component. All the available evidence, however, indicates that different lysins react with the same cell component, probably the protein "stromatin" [see Ponder *et al.*, 1930], and they certainly all react with that component which is responsible for the discoidal form [Ponder, 1934]. We have accordingly no collateral evidence supporting possibility (*a*), and, while we cannot altogether disprove it, we can conclude that it is not a very likely one.

We have therefore to fall back upon the second explanation for the results obtained in the above tables, *viz.* that the additive effects of two lysins are usually obscured by various inhibitory and acceleratory phenomena which result from an interaction of the lysins with each other.

Summary.

When two simple lysins (saponin, digitonin, sodium taurocholate, sodium glycocholate or sodium oleate) act together in a system, the total activity is rarely the same as the sum of the two individual haemolytic activities. The most

likely explanation of the result is that the lysins interact with each other, thereby mutually enhancing or inhibiting each other's lytic activity.

Some curious difficulties arise in the analysis of the combined effects of two lysins, and these are discussed at some length.

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LIII. THE HYDROLYSIS OF CERTAIN POLYSACCHARIDES AND PROTEINS BY THE ENDOSPORES OF AEROBIC BACILLI.

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A study of the enzymic content of the endospores of some twelve unidentified aerobic bacilli was made by Ruehle [1923]. He found that the spore preparations studied by him liquefied gelatin which contained 0·5 % of phenol and was covered by toluene, and this observation was confirmed by Cook [1931]. Ruehle also investigated the ability of certain of his preparations to hydrolyse fats and caseinogen and obtained negative, or at the best feebly positive, results. In a previous paper [Tarr, 1932] it was shown that almost every cell in a given culture forms spores when the aerobic bacillus being studied is cultivated under appropriate conditions. Later [Tarr, 1933] it was found that endospores of aerobic bacilli possess active respiratory catalysts which are quite different from the respiratory catalysts of the vegetative cells from which they are formed. In the present communication it is shown that endospores of aerobic bacilli possess an amylase and proteases.

Experimental.

The cultures employed (*B. subtilis* I; *B. subtilis* II and *B. mesentericus* I) were identical with those used in previous work [Tarr, 1932]. Endospore suspensions were prepared by the method previously described [Tarr, 1933] and were heated to 80° for 30 minutes immediately prior to use.

The amylase of bacterial endospores.

The presence of an amylase in bacterial endospores was verified by studying the hydrolysis of both starch and glycogen in the presence of toluene as antiseptic in the following manner. 250 ml. Erlenmeyer flasks received 50 ml. of 0·5 % solution of soluble starch (or glycogen), 10 ml. of $M/5$ phosphate buffer p_H 7·3, 15 ml. of water and 50 ml. of endospore suspension. The liquid in each flask was covered with 10 ml. of toluene, and the flask was corked and then incubated at 37°, shaking well each day. At suitable intervals portions of the solutions were removed for examination, the hydrolysis being followed in three ways: (1) the colour formed when a drop of the solution was mixed with a drop of $N/100$ iodine was noted; (2) the relative ability of 1 ml. of the experimental solution to reduce a drop of Benedict's solution was determined; (3) the amount of aldose present in a 5 ml. sample was estimated by the method of Willstätter and Schudel [1918], the results being reported in ml. of $N/100$ iodine per 5 ml. of solution.

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The results of this experiment which are recorded in Tables I and II show that heated spores of the organisms studied hydrolyse both starch and glycogen in the presence of toluene, for the typical reaction of the polysaccharide with iodine disappears in each case, the amount of reducing sugar increases, and the aldose titre increases. That the reducing aldose formed in each case is maltose and not glucose is shown by the following experiment.

Table I. *Hydrolysis of starch by bacterial endospores.*

Time in days	Iodine test			Reducing sugar test			Aldose formed (ml. *N*/100 I)		
	B. subtilis I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*
0	Blue	Blue	Blue	–	–	–	0·28 0·29	0·29 0·29	0·29 0·29
2	Red-brown	Blue	Red-brown	+	+	–	—	—	—
3	Red-brown	Red-brown	Neg.	+	++	–	—	—	—
5	Red-brown	Neg.	Neg.	++	+++	+	0·54 0·56	0·53 0·53	0·42 0·42
9	Neg.	Neg.	Neg.	+++	+++	+++	0·64 0·64	0·66 0·66	0·61 0·61
14	Neg.	Neg.	Neg.	+++	+++	+++	0·64 0·64	0·68 0·70	0·65 0·66

B. subtilis I 280 mg. dry wt. per exp.
B. subtilis II 480 ,,
B. mesentericus 410 ,,

– No reduction.
+ Slight reduction.
++ Fair reduction.
+++ Pronounced reduction.

Table II. *Hydrolysis of glycogen by bacterial endospores.*

Time in days	Iodine test			Reducing sugar test			Aldose formed (ml. *N*/100 I)		
	B. subtilis I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*	*B. subtilis* I	*B. subtilis* II	*B. mesentericus*
0	Red-brown	Red-brown	Red-brown	–	–	–	0·28 0·29	0·30 0·30	0·30 0·30
2	Neg.	Almost neg.	Almost neg.	+	–	–	—	—	—
3	Neg.	Neg.	Neg.	+	+	–	—	—	—
5	,,	,,	,,	++	+++	+	0·48 0·50	0·46 0·48	0·35 0·35
9	,,	,,	,,	+++	+++	+	0·64 0·64	0·48 0·46	0·36 0·36
14	,,	,,	,,	+++	+++	+++	0·64 0·64	0·48 0·48	0·48 0·48

B. subtilis I 280 mg. dry wt. per exp.
B. subtilis II 480 ,,
B. mesentericus 410 ,,

– No reduction.
+ Slight reduction.
++ Fair reduction.
+++ Pronounced reduction.

After the conclusion of the above experiment 25 ml. of the remaining liquid in each flask were filtered through kieselguhr, the clear filtrate was evaporated at reduced pressure to about 3 ml. and the osazone prepared following the directions given by Cole [1933]. In each case typical maltosazone crystals appeared on cooling the liquid. Attempts to hydrolyse 0·2 % maltose solutions with endospore suspensions under toluene were unsuccessful; no increase in the aldose titre was obtained after 2 weeks at 37°.

The proteolytic activity of endospores.

The ability of bacterial endospores to hydrolyse various proteins was next investigated. 2 % solutions or suspensions of the proteins studied were prepared. 10 ml. of such a solution were placed in a 50 ml. Erlenmeyer flask together with

5 ml. of $M/5$ phosphate buffer p_H 7·3, 5 ml. of spore suspension and 2 ml. of toluene. The flasks were corked and incubated at 37°, shaking thoroughly each day, and 1 ml. samples were removed at intervals for formaldehyde titration by the method of Brown [1923]. The results of this experiment which are given in Table III show that bacterial endospores hydrolyse caseinogen, gelatin, egg-albumin, blood-albumin, peptone (Witte), and, in the case of one species, edestin, the number of free amino-groups in the solution increasing in each case. No increase in the initial formaldehyde titration value was obtained when endospores or proteins alone were incubated under similar conditions.

Table III. *Hydrolysis of proteins by bacterial endospores.*

No. of ml. of $N/20$ NaOH required for 1 ml. of the following protein solutions using the formaldehyde titration method of Brown.

Time in days	Caseinogen		Egg-albumin		Blood-albumin		Edestin		Gelatin		Peptone (Witte)	
	B. subtilis I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*	*B. subtilis* I	*B. mesentericus*
0	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·08	0·18	0·18
7	0·14	0·10	0·08	0·08	0·12	0·09	0·07	0·05	0·10	0·06	0·22	0·20
14	0·19	0·12	0·12	0·10	0·21	0·10	0·08	0·06	0·16	0·14	0·34	0·26
21	0·18	0·14	0·11	0·13	0·20	0·10	0·12	0·05	0·20	0·14	0·33	0·34

The results given are the averages of duplicate experiments agreeing within 5 %.

B. subtilis I 66 mg. dry weight per exp.
B. mesentericus 42 ,,

Summary.

Endospores of aerobic bacilli hydrolyse starch and glycogen to maltose, and various proteins with an increase in the number of free amino-groups in the solution.

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LIV. FAT METABOLISM IN FISHES.
III. SELECTIVE FORMATION OF FAT DEPOSITS.

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In a previous communication [Lovern, 1932, 2] data were given of the fatty acid compositions of the glycerides from three depôts in a single fish (*Acipenser sturio*), and some evidence was adduced that a reversible change from palmitic into palmitoleic acid was one factor controlling depôt fat composition. It was also suggested that for the sturgeon the fat in all depôts required a constant ratio of saturated to unsaturated acids. Three other species have now been studied, the conger eel (*Conger vulgaris*), a dolphin (species unfortunately not determined with certainty), and a porpoise (*Phocaena communis*). In the case of the conger eel, the main fat depôt consists of two strips of adipose tissue attached to the peritoneum, whilst the liver is a subsidiary depôt. In order to obtain sufficient liver fat for a full analysis, twelve large fish were used, the peritoneal fats and liver fats being combined. In the case of the dolphin (an immature specimen) and the porpoise (an adult female with a well-developed foetus) the main fat depôt is the blubber. This was divided into two classes in each case; (*a*) that from the body, and (*b*) that from the head, which is mainly found in a thick deposit in a hollow of the skull. These two fats had different compositions. In addition, the jawbones of these animals are filled with an oily pulp, which was extracted, giving a fat of different composition from the blubber fats.

In the hope of extending the range of depôts examined, a semi-micro-method of fatty acid analysis was elaborated. As pointed out previously [Lovern, 1932, 2], for an accurate analysis of a fish oil by macro-methods, roughly 200 g. of fat are required. It was desired to carry out such an analysis on quantities down to 10 g. of fat. A small distilling flask was used, with a bulb of 50 ml. capacity and a side-tube let into the bulb as in the Willstätter flask. A slight constriction in the neck supported a spiral of copper wire, to act as a fractionating column. Quantities as small as 1 g. could be fractionated from this, the residues usually being no more than 0·4 to 0·5 g. Fractions were collected in receivers made from shortened test-tubes, and quantities as low as 0·3 g. were frequently collected and the saponification equivalent and iodine value determined. Iodine values on one quarter of the usual quantity of material are easily performed by the use of proportionately smaller quantities of reagents and titration with more dilute thiosulphate. Equivalent determinations on as little as 0·1 g. of material were carried out. The fat was weighed into a shortened test-tube, 2 ml. of $N/2$ alcoholic potash were added and the mixture was boiled for one hour under reflux with exclusion of carbon dioxide. The contents were washed out with 80 ml. of neutral alcohol into a suitable flask and titrated with $N/50$ sulphuric acid. A good end-point at such dilution depends on the choice of a suitable indicator, *e.g.* dibromothymoltetrachlorophthalein [Cornwell and Esselstyn, 1927]. This has a

very small p_H range (8·8 to 8·4) from deep blue to colourless, and after a little practice, satisfactory duplicates could always be obtained. A blank determination was made as usual.

In every case, as large a quantity of fat as possible (up to 200 g.) was used. Wherever possible, fractional distillations were carried out in the usual Willstätter flasks, and where this was impossible, in the small flask. Equivalent and iodine value determinations were also performed on full quantities where available, and when determined on smaller amounts, the procedure was adapted to make use of as much material as was available. Unfortunately, in the micro-method, it was often impossible to refractionate the fractions from the primary distillation, and this is probably the greatest source of error. In certain cases mixtures have to be taken as containing only two components for the composition to be calculated, and it is almost impossible to obtain only such fractions in one distillation, especially perhaps in this simpler distilling flask. Each fraction however will contain mainly two components, and in many cases only two.

By the use of these micro-methods, analyses were also made of the fats from the blubber of the foetus, and the liver, lungs and heart of the porpoise. In each case only the glyceride fraction was used, the accompanying phosphatides being separated with acetone. The analytical procedure was the same as that adopted in previous cases, except as regards the *iso*valeric acid, which was recovered by steam-distillation. Small quantities of lauric acid also always came over at the same time. The steam-volatile acids were dealt with by ether extraction and fractional distillation, as in the method of Hilditch and Jones [1929], for butter-fats.

The blubber and jaw-fats of the dolphin and porpoise all contain small amounts of waxes, but glycerides preponderate to such an extent that the presence of the waxes can be ignored and the mixed fatty acid composition considered as being essentially that of the glyceride fatty acids. In one case only, that of the head blubber of the dolphin, was the higher alcohol mixture from these waxes examined in detail. It was found to consist mainly of cetyl and oleyl alcohols, with appreciable amounts of tetradecanol and octadecanol. The method used in this analysis was that described by Hilditch and Lovern [1929, 2].

The blubber fats from the members of the porpoise and dolphin family are well-known to be peculiar in respect of the presence of *iso*valeric acid. Gill and Tucker [1930] found in the jaw-oil of a species of dolphin (*Tursiops truncatus*) 86·7 % *iso*valeric acid, 8·4 % palmitic acid and 4·9 % oleic acid. They also found 18·7 % of higher alcohols (mainly dodecanol with some tetradecanol). It must be pointed out that their deduction as to the palmitic and oleic acid percentages was based only on an equivalent and iodine value of the mixed non-steam-volatile acids. By analogy with the results of the present analyses, it seems most improbable that only these two acids were present; indeed, one would expect myristic and palmitoleic to be more important constituents than palmitic and oleic, since the percentage of *iso*valeric acid was exceptionally high, and, in the present results, the higher the percentage of *iso*valeric acid, the larger the proportions of myristic and palmitoleic acids.

The analyses are given in Tables I, II and III, Table I giving the particulars of the oils, and Tables II and III the quantitative compositions of the mixed fatty acids, as weight and molecular percentages respectively.

As in previous papers, the average degree of unsaturation of the C_{18}, C_{20} and C_{22} acids is expressed in terms of lack of hydrogen. The C_{14} and C_{16} unsaturated acids were in every case monoethylenic only. The palmitoleic and oleic acids in each fat were the ordinary $\Delta^{9,10}$ acids, as shown by the production of azelaic acid on oxidation with permanganate in acetone solution.

Table I. *Particulars of fats.*

Species	Fat from	Iodine value	Sap. equiv.	Unsaponifiable %	Type of unsaponifiable
Conger vulgaris	Liver	109·4	—	2·5	Mainly cholesterol
,,	Peritoneum	141·7	—	0·7	,,
Dolphin	Body blubber	136·0	263·4	2·2	Mainly higher alcohols
,,	Head blubber	82·33	228·4	7·5	,,
Phocaena communis	Body blubber	88·82	225·7	2·4	,,
,,	Head blubber	64·76	204·7	2·1	,,
,,	Jaw	44·93	196·3	3·6	,,
,,	Foetal body blubber	108·9	267·8	2·4	Largely cholesterol
,,	Heart	121·3	—	9·7	Mainly cholesterol
,,	Lungs	119·5	—	15·0	,,
,,	Liver	175·0	—	32·1	,,

Table II. *Composition of mixed fatty acids (Wt. %).*

		Saturated					Unsaturated				
Species	Depôt	C_8	C_{12}	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Conger vulgaris	Liver	—	—	5·2	19·2	0·4	0·4	18·0	37·5 (−2·0H)	12·4 (−5·6H)	6·9 (−8·1H)
,,	Peritoneum	—	—	1·8	18·8	0·9	—	6·2	40·6 (−2·1H)	18·3 (−5·4H)	13·4 (−8·4H)
Dolphin	Body	3·2	1·0	7·2	8·6	0·8	4·7	25·9	24·1 (−3·3H)	18·6 (−6·5H)	5·9 (−7·6H)
,,	Head	13·9	2·4	12·5	11·6	0·4	2·7	25·4	15·8 (−2·8H)	12·7 (−5·5H)	2·6 (−7·2H)
Phocaena communis	Body	13·6	3·5*	12·1	4·7	—	4·7	27·2	16·7 (−2·8H)	10·5 (−4·8H)	7·0 (−4·9H)
,,	Head	20·8	4·1	15·8	7·5	0·2	4·6	20·8	15·2 (−2·6H)	9·4 (−4·5H)	1·6 (−4·7H)
,,	Jaw	25·3	4·6*	28·3	4·1	—	3·2	20·3	9·3 (−2·6H)	4·9 (−4·9H)	—
,,	Foetus	1·2	—	14·9	0·6	—	12·3	48·1	15·4 (−4·0H)	7·5 (−7·4H)	—
,,	Liver	—	—	—	7·6	5·5	—	6·1	42·5 (−2·8H)	27·3 (−5·4H)	11·0 (−6·5H)
,,	Lungs	—	—	4·6	9·0	1·2	0·1	16·5	27·0 (−2·4H)	31·0 (−3·3H)	10·6 (−5·4H)
,,	Heart	—	—	8·1	8·2	4·4	4·4	16·8	50·4 (−3·6H)	7·6 (−5·4H)	—

* Trace of lauroleic acid present.

Table III. *Composition of mixed fatty acids (Mols. %).*

		Saturated					Unsaturated				
Species	Depôt	C_8	C_{12}	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Conger vulgaris	Liver	—	—	6·2	20·5	0·4	0·5	19·4	36·3	11·0	5·7
,,	Peritoneum	—	—	2·3	20·8	0·9	—	6·9	40·8	16·9	11·4
Dolphin	Body	8·0	1·3	8·1	8·6	0·7	5·3	26·1	21·9	15·5	4·5
,,	Head	29·2	2·6	11·7	9·7	0·3	2·5	21·4	12·0	8·9	1·7
Phocaena communis	Body	28·7	3·8	11·4	3·9	—	4·5	23·0	12·8	7·4	4·5
,,	Head	39·6	4·0	13·4	5·7	0·1	3·9	16·0	10·5	5·9	0·9
,,	Jaw	44·7	4·1	22·4	2·9	—	2·6	14·4	6·0	2·9	—
,,	Foetus	2·9	—	16·2	0·6	—	13·5	47·0	13·7	6·1	—
,,	Liver	—	—	—	8·6	5·6	—	6·9	43·7	25·7	9·5
,,	Lungs	—	—	5·7	10·0	1·2	0·1	18·4	27·2	28·4	9·0
,,	Heart	—	—	9·5	8·6	4·2	5·2	17·7	48·2	6·6	—

Consideration of these figures reveals several points of interest. In the conger eel we have another instance, as in the sturgeon, of an animal with a liver fat no more unsaturated than the depôt fat. But whereas in the sturgeon both fats have almost equal average unsaturations (iodine value), in the conger eel the liver fat has a much lower iodine value and is much harder. This is wholly due to the different proportions of the various acids present in each case. Unlike the sturgeon, the conger eel has no constant proportion of saturated acids. The oils resemble that from the liver of the halibut in the absence of linoleic acid [Lovern, 1932, 1].

The acids from the dolphin and porpoise blubber and jaw depôts resemble those of the oils from the sperm whale [Hilditch and Lovern, 1928], particularly the head oil of this whale, in that in each case a considerable proportion of the acids have molecular weights below those of the acids taken in the food and are hence products of metabolism. Also the proportions of certain acids which are present in the food are much altered in these depôts, *e.g.* the high proportions of myristic and palmitoleic acids. The oils further resemble the sperm oils in being a mixture of waxes and glycerides, although the proportion of waxes is much lower in the present cases. The occurrence of *iso*valeric acid as a fat constituent is apparently peculiar to the Delphinidae, but lauric acid has been found elsewhere in marine animals [Rees, 1933; Hilditch and Lovern, 1928].

The foetal fat of the porpoise had only a trace of *iso*valeric acid, but large quantities of C_{14} and C_{16} acids, whilst the degree of unsaturation of the C_{18} and C_{20} acids was the highest in the whole series, considerably higher even than that of the maternal liver. The liver fat had the highest average unsaturation, as shown by the iodine value, but this is due largely to the proportions of the different groups of acids (contrasted with the heart fat for instance). It is noteworthy that the organ fats have no *iso*valeric and lauric acids and do not contain unusually large proportions of C_{14} and C_{16} acids. In this respect also the porpoise and dolphin resemble the sperm whale, for Tsujimoto and Kimura [1928] reported the liver fat of the latter to be only glycerides, and with no preponderance of lower acids. This difference from the blubber and jaw fats is particularly emphasised when the results are expressed in molecular percentages.

If the *iso*valeric, lauric and much of the myristic acids, which are presumably metabolic products, had been separately built up into glycerides and then admixed with food fat entering the depôts, we should expect most of these acids to be present as fully-saturated glycerides, which would survive oxidation with powdered permanganate in acetone solution. The body fat of the porpoise which was used contained 10·5 % by weight of fully-saturated material. This was for the most part glycerides, although a small amount of higher alcohols was recovered from it. The composition of the mixed acids is given (wt. %) in Table IV. The significance of these results is best seen by comparing the proportions of each acid present as fully-saturated material.

Table IV. *Proportions of saturated acids combined as fully-saturated glycerides.*

	C_8	C_{12}	C_{14}	C_{16}
Composition of acids in fully-saturated portion	26·1	19·7	42·1	12·1
Reduced to 10·5 total	2·7	2·1	4·4	1·3
Present in 100 parts of original fat	13·6	3·5	12·1	4·7
% of original acid appearing as fully-saturated glycerides	19·9	60·0	36·4	27·7

The results for the lauric, myristic and palmitic acids are in full agreement with those for sperm head oil [Hilditch and Lovern, 1929, 1] in that larger proportions of the lower molecular weight acids are combined as fully-saturated material, but the *iso*valeric acid is an outstanding exception. Only 20 % of it is so combined, the remainder being combined in glycerides which have at least one unsaturated acid in the molecule. That 80 % of the *iso*valeric acid is combined as mixed saturated-unsaturated glycerides was proved by eliminating the two alternative explanations of the oxidation results. One of these is that *iso*valeric acid is itself unstable to oxidation. This was disproved by a second oxidation, which gave the same quantity of fully-saturated material, of the same mean equivalent. The second alternative is that the bulk of the *iso*valeric acid is present as mono- or di-glycerides, which would yield acidic products on oxidation. Gill and Tucker [1930] suggested that in the jaw fat of *Tursiops truncatus* some of it was so combined. A sample of the porpoise body fat was acetylated, and the mean equivalent of the product was 220·0 as against 225·7 before acetylation. This fall is easily accounted for by slight rancidity and the presence of traces of free alcohols, cholesterol, *etc.*, and it is evident that no great quantity of mono- or di-glycerides can be present.

Whatever the origin of the *iso*valeric acid (and this may well be the same as that by which small quantities of this acid are normally produced as waste products in many animals), it can hardly be the same as that of the other fatty acids, all of which have a straight chain structure. The members of the Delphinidae differ from all other animals yet investigated in using this *iso*valeric acid as a constituent of their depôt fats, and it is perhaps also the only case of an acid of an odd number of carbon atoms being so used. In this utilisation of the *iso*valeric acid, it is largely combined as mixed glycerides with ordinary *n*-fatty acids, and the mode of combination is quantitatively quite different from that of the other saturated acids. In the case of the foetus it appears that *iso*valeric acid was not passed through the placenta, and that the portion found in the foetal blubber was produced by metabolic processes in the foetus itself. A small amount of fat was isolated from the placenta and the glyceride and phosphatide fractions separated. No *iso*valeric acid was present, and the glyceride acids had an iodine value of 117·7 and a mean equivalent of 275·5. Hence no large proportions of C_{14} and C_{16} acids could have been present. The acids from the phosphatide fraction had an iodine value of 96·7, too little being available for the determination of the mean equivalent.

A comparison of the fats from the depôts of each animal (excluding for the moment the organ fats, which are not really depôt fats, and the foetal blubber, which really belongs to a separate metabolic system) shows that the differences are progressive with the molecular weight of the component acids. For instance, in *Phocaena communis* (Table III), on passing from body to head and head to jaw, the *iso*valeric, lauric and myristic acids steadily rise, and the C_{18}, C_{20} and C_{22} acids fall. If the compositions are expressed as total groups (*i.e.* all C_{16} acids being grouped together for instance) in molar percentages, this progressive effect is striking (Table V).

In the conger eel, C_{14} and C_{16} acids are less in the peritoneum than in the liver, and C_{18}, C_{20} and C_{22} are higher. For the dolphin and porpoise the C_5, C_{12} and C_{14} acids increase from body to head and head to jaw, whilst the C_{16}, C_{18}, C_{20} and C_{22} acids decrease. That the extent of these increases and decreases is in general progressive with molecular weight is apparent. The figures for the acids from the head and body blubbers of the sperm whale [Hilditch and Lovern, 1928] show precisely the same relationship.

Table V. *Acids arranged in molecular groups.*

Species	Depôt	C_5	C_{12}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
Conger vulgaris	Liver	—	—	6·7	39·9	36·7	11·0	5·7
,,	Peritoneum	—	—	2·3	27·7	41·7	16·9	11·4
Dolphin	Body	8·0	1·3	13·4	34·7	22·6	15·5	4·5
,,	Head	29·2	2·6	14·2	31·1	12·3	8·9	1·7
Phocaena communis	Body	28·7	3·8	15·9	26·9	12·8	7·4	4·5
,,	Head	39·6	4·0	17·3	21·7	10·6	5·9	0·9
,,	Jaw	44·7	4·1	25·0	17·3	6·0	2·9	—

This suggests that one of the mechanisms operating in the case of these animals to control the type of fat laid down in the different depôts rests on a basis of molecular size and might almost be some form of molecular filtration. Presumably the acids enter the depôts as glycerides and not as free fatty acids, but in either case similar reasoning could be applied. We will suppose for example, that in *Phocaena communis* the size of molecule which can readily pass through the membranes of the jaw depôt is less than that for the head, which in turn is less than that for the body. Under such circumstances a selective deposition of certain of the available glycerides would take place exactly in accordance with Table V. In the less permeable jaw depôt for instance, C_{20} acids could only enter if combined as a triglyceride with two molecules of *iso*valeric acid, and C_{22} acids could not enter at all. More combinations of C_{18} acids would find an entry, *e.g.* one C_{18} radical with two C_5 or two C_{12} radicals, or with one C_5 and a C_{12} or C_{14} radical, and so on progressively for the other acids. What becomes of much of the higher molecular weight acids which the porpoise and dolphin take in their food (this applies also to the sperm whale) is not clear. A small amount will find its way into the organ fats in which lower acids do not preponderate, but it seems not unlikely that much of it is broken down into these same lower acids which are present in such large amounts. The somewhat low degree of unsaturation of the porpoise, dolphin and sperm whale C_{20} and C_{22} acids may be of significance in this respect. This progressive difference in the fat from the different depôts is also shown qualitatively by the characteristics of the fats from the same three depôts of another dolphin (*Lagenorhynchus albirostris*).

Table VI. *Fat from* Lagenorhynchus albirostris.

Depôt	Saponification equivalent	Iodine value
Body	253·9	112·2
Head	246·9	99·81
Jaw	226·1	65·15

Sunderland [1932] examined fats from *Phocaena communis*, making a different separation of the depôts. His samples were from the upper and lower jaws respectively, from the skull and from the blubber. The blubber included both the head and body sections. The fats had different saponification and iodine values, again showing a selective deposition.

If we visualise fat deposition taking place selectively on any basis of permeability differences, we should expect to find, *mutatis mutandis*, that the less permeable depôts (as found by analysis of the fatty acids) would be the smaller in size. Low molecular weight components will find equally easy entry into all depôts, but the larger molecules will only readily enter the more permeable depôts. In actual fact, such is the case. In the conger eel, the less permeable liver depôt contains roughly one-eighth as much fat as the peritoneal depôt. In the dolphin and porpoise, the body fat is much greater in quantity than the head

fat, which in turn is much greater than the jaw fat. The writer has no data for the relative quantities of head and body fat in the sperm whale, but it seems most probable that the body fat is the greater in quantity.

The position in the sperm whale is complicated, however, by the presence of so much wax, which is mainly of low molecular weight compared with triglycerides. Whilst this evidence as to relative size of depôts is in harmony with the molecular filtration theory, it must be borne in mind that some depôts may be restricted by anatomical structure, *e.g.* the jaw depôt of the porpoise. There is also the evidence of Channon and El Saby [1932] that in the herring fat does not necessarily enter all the depôts at the same time and may even be transferred from one depôt to another. It may well be that similar conditions obtain in the animals under discussion.

On this theory of molecular filtration, the saturated and unsaturated acids of the same number of carbon atoms would stand equal chances of entering any particular depôt, unless, of course, there is something peculiar in the mode of combination as glycerides. The only glyceride peculiarity so far evident which bears on this point is the tendency for lower saturated acids to concentrate in the fully-saturated fraction of the fat. Since all the saturated acids present are of relatively low molecular weight, this might conceivably result in a greater chance of saturated acids being found in small glyceride molecules than the corresponding unsaturated acids, and this would be more marked for myristic than for palmitic acid (Table IV). Had the glyceride composition been quite heterogeneous, one would have expected to find the same ratios of palmitic to palmitoleic acid and of myristic to myristoleic acid entering a depôt as were present in the supplies reaching that depôt, and hence the same *ratio* in each depôt, irrespective of the total quantities of these acids. The ratios are given in Table VII. In the case of the conger eel only the C_{16} ratio can be included.

Table VII. *Ratio of saturated to unsaturated acid.*

Species	Depôt	C_{14} acids	C_{16} acids
Conger vulgaris	Liver	—	1·1
,,	Peritoneum	—	3·0
Dolphin	Body	1·5	0·33
,,	Head	4·6	0·46
Phocaena communis	Body	2·6	0·17
,,	Head	3·4	0·36
,,	Jaw	8·8	0·20

The differences in the ratios are very large in certain cases, especially for the conger eel and for C_{14} acids in the dolphin and porpoise. With one exception the differences for the dolphin and porpoise are all in the same direction, an increasing ratio of saturated to unsaturated acid on passing from body to head and head to jaw. This is in agreement with the glyceride peculiarity. The jaw of the porpoise is an exception for the C_{16} acids, and in the conger eel there is a big difference in the opposite direction, *i.e.* a higher ratio in the more permeable depôt, which cannot be explained by glyceride structure.

The interpretation which seems most in accordance with all the facts is that the saturated and unsaturated acids of the same number of carbon atoms are interconvertible, as suggested in the previous paper for the sturgeon [Lovern, 1932, 2]. It would follow that the conversion takes place in the depôt itself. Support of the idea of interconvertibility is found in the work of Mazza and Stolfi [1933] and of Berend [1933] who have described dehydrogenases which act on higher fatty acids.

The peculiar composition of the porpoise and dolphin depôt fats raises some interesting questions. Where, for instance, is the site of the production of the lower fatty acids, due perhaps to breakdown of the C_{20} and C_{22} acids? Also of the *iso*valeric acid, which is apparently produced quite separately, and combined into mixed glycerides in a different manner from the other lower acids? That this site is the liver seems most unlikely, in view of the apparent entire absence of these acids from it—indeed, the liver fat closely resembles the other organ fats examined. In fact, the organ and the depôt fats are so thoroughly different in their whole composition that it is difficult to imagine the liver playing any large part in the metabolism of these depôt fats. An organ that is worthy of attention in this respect is the spleen, in view of its function of removing fat from the blood during alimentary lipaemia [Marino, 1933], and hence its rôle as an intermediary in the formation of depôt fats from food fat [*cf.* also Leites *et al.*, 1933].

In the case of the foetus it seems probable that the fat received from the maternal system is mainly of the ordinary marine type. This is modified by the foetus in two ways—by production of much C_{14} and C_{16} acids in place of C_{20} and C_{22}, and by admixture of *iso*valeric acid. The metabolic process from which the *iso*valeric acid arises evidently does not function to any great extent up to this stage. The characteristic composition of the foetal fat is further evidence that the *iso*valeric acid and the lower *n*-acids are produced by separate processes.

The molecular selectivity displayed in the formation of these depôt fats does not appear to hold for all species, *e.g.* the pike and the sturgeon [Lovern, 1932, 1, 2]. In these animals the different depôts appear equally permeable, resulting in the constant proportion of total C_{16} acids found for the sturgeon and the pike.

Summary.

The liver and peritoneal fats of the conger eel, and the fats from a number of depôts and organs of a dolphin and a porpoise have been examined. Considerable differences in fatty acid composition are found in the different fats from one animal, indicating a highly selective formation of the fat deposits.

The porpoise and dolphin fats show great similarity of type to those from the sperm whale, thus affording further evidence that zoologically related species exhibit definite similarities in fat composition.

The porpoise and dolphin depôt fats are unique in containing large amounts of *iso*valeric acid and the mode of combination of this is peculiar.

The depôt fats of the porpoise and dolphin are quite different in type from the organ fats of the same animals. Whilst no explanation of this can be offered, it is not in harmony with the idea of fat transfer to the liver before utilisation. The theory of desaturation in the liver is also unsupported by the conger eel and porpoise figures. In the case of the porpoise, the heart fat contains an even more unsaturated group than the liver fat.

The blubber of a porpoise foetus showed a peculiar composition and a very high degree of unsaturation.

The depôt fats of all the animals show a molecular size gradation which suggests that one cause of specificity may be a molecular filtration.

Some further evidence is adduced in support of the idea of interconvertibility of saturated and unsaturated acids of the same number of carbon atoms.

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LV. A NOTE ON THE STRUCTURE AND CHEMISTRY OF GLUCURONIC ACID MONOBENZOATE.

By ARMAND JAMES QUICK.

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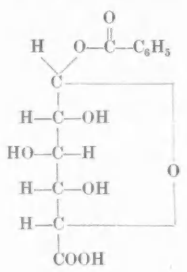
(*Received January 25th, 1934.*)

Although glucuronic acid was discovered over 50 years ago, its position in physiology remained until recently undefined. Now, however, it seems fairly certain that this compound is intimately related to carbohydrate metabolism. The depancreatised dog can still produce glucuronic acid from the glycogenetic fraction of the protein molecule, which would otherwise go to glucose and be excreted [Quick, 1926, 2]; furthermore, insulin can markedly increase while acetoacetic acid as well as lactic acid decidedly suppress its output [Quick, 1932]. The importance of glucuronic acid depends not only on its relationship to carbohydrates, but also on the fact that it represents a type of conjugation, which is commonly referred to as a detoxication mechanism. In the opinion of the writer these synthetic processes are constantly active in normal metabolism, but they often only become manifest when the organism applies them to abnormal substances. It is probable that the uric acid-carbohydrate compound normally present in the blood is produced by a conjugating mechanism and may represent an important step in the excretion of uric acid.

The desirability of studying the molecular structure and chemistry of the various conjugated glucuronic acids is evident. Recently Pryde and Williams [1933] reported studies on the structure of bornyl-*d*-glucuronide, glucurone, and the glucuronic acid-benzoic acid compound. This last compound was prepared and studied by Quick [1926, 1]. From the data then obtained, he concluded that the compound was not benzoylglucuronic acid as Magnus-Levy [1907] had postulated (I) but glucuronic acid monobenzoate (II).



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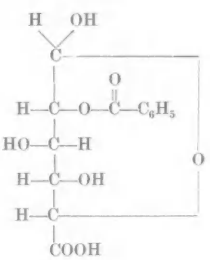

II

Pryde and Williams, however, question the ester structure and believe that the formula originally proposed by Magnus-Levy is the correct one. The writer's contention that the compound has formula II is based in part on the finding that sodium cyanide reacts directly with glucuronic acid monobenzoate, thus indicating that the aldehyde group is free and is not combined with benzoic acid. Although Pryde and Williams obtained the same polariscopic changes as the author, they claim that the alkalinity of the sodium cyanide first caused a hydrolysis, and that it was the liberated glucuronic acid which reacted. They base their conclusions on the qualitative detection of benzoic acid, which obviously can be misleading, and was unfortunate in view of the fact that satisfactory quantitative methods were available, which would have prevented a hasty and erroneous conclusion.

In order to redemonstrate that sodium cyanide reacts with glucuronic acid monobenzoate directly without previous hydrolysis, the following experiment was performed.

0·4 g. of glucuronic acid monobenzoate was dissolved in 20 ml. of water and mixed with a solution containing 0·09 g. sodium cyanide in 20 ml. The resulting mixture was faintly alkaline to phenolphthalein and became progressively more so as the reaction progressed. At the end of 21 hours, the solution was acidified with hydrochloric acid until acid to Congo red, then extracted with 4 portions (25, 25, 15, 10 ml.) of neutral toluene and the benzoic acid determined in the usual manner by titration with 0·1 *N* sodium ethoxide. The amount of free benzoic acid found was 0·0145 g. while the total amount contained in 0·4 g. of glucuronic acid monobenzoate is 0·1635 g. Therefore only 8·9 % of the compound was hydrolysed in 21 hours. At the same time, a determination of the reducing action made on an aliquot by the method of Shaffer and Hartmann [1920–21] showed that only 3·9 % of the original reducing power remained, indicating that 96·1 % of the aldehyde had reacted to form the cyanohydrin derivative. It must be concluded that the benzoic acid is not attached to the aldehyde group, for, if it were, an amount of benzoic acid equivalent to the quantity of cyanohydrin produced should be formed. Therefore the benzoic acid must be linked to one of the hydroxy-groups of glucuronic acid.

Although the experiment presented answers the criticism of Pryde and Williams there are sufficient data in the writer's original paper to support adequately the claim that the compound under discussion is not benzoylglucuronic acid but a glucuronic acid monobenzoate.

In the first place, when 0·2 g. of glucuronic acid monobenzoate (which requires 0·033 g. of sodium cyanide) was treated with a slight excess, *i.e.* 0·045 g. of sodium cyanide, the solution was alkaline to phenolphthalein and became more alkaline as the reaction progressed. If hydrolysis had occurred, the solution should have become acid, since the amount of benzoic acid liberated would have greatly exceeded the amount of base available for neutralisation.

In the second place, glucuronic acid monobenzoate showed mutarotation in an alkaline solution. As the writer expressly stated, this change of rotation occurred with scarcely any hydrolysis.

Further study is required to determine by which hydroxy-group the benzoic acid is linked to glucuronic acid. Probably the most promising approach to the problem is the preparation of the methyl ester methylglucoside, as described by the writer, before attempting complete methylation.

SUMMARY.

The compound resulting from the conjugation of glucuronic acid with benzoic acid is a glucuronic acid monobenzoate, and not benzoylglucuronic acid, for it acts directly with sodium cyanide to form the cyanohydrin, and it exhibits mutarotation.

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LVI. THE RELATION BETWEEN THE ACTIVITY OF UREASE AND THE OXIDATION-REDUCTION POTENTIAL.

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(*Received January 30th, 1934.*)

Amongst the substances which exercise accelerating or inhibitory actions on the activity of enzymes, particular interest has recently been attached to compounds possessing reducing or oxidising properties such as hydrogen sulphide, cysteine, glutathione, iron and other metallic complexes, and more recently also ascorbic acid [Karrer and Zehender, 1933; Edlbacher *et al.*, 1933, 1, 2]. These substances may react chemically with the enzyme-substrate system or they may influence the enzyme reaction by stabilising the oxidation-reduction potential of the medium. In the first case the activity will be related to the particular properties of the system added, whilst in the latter case every oxidation-reduction system will maintain the same potential and have the same influence. Mainly Waldschmidt-Leitz *et al.* [1929; 1930; 1933] and Grassmann *et al.* [1930; 1930–31] have indicated a relationship between the oxidation-reduction potential and the activity of the enzyme. Obviously a method of elucidating this problem would be to investigate the activity of the enzyme in the presence of different oxidation-reduction systems which maintain the potential (r_H) at known levels but which do not react chemically in any other way with the constituents of the mixture—in other words to impose conditions similar in character to those employed to maintain a desired hydrogen ion concentration in a reaction mixture.

There is however a difficulty in that a very large number of different oxidation-reduction systems are necessary in order to cover the entire range of the r_H scale. Each of the common oxidation-reduction systems is suitable only for a range of about 4 r_H units, whereas the full range between the hydrogen and oxygen electrodes is about 40 units. For this reason approximately ten systems are necessary in order to obtain a complete curve covering relation between activity and r_H. However, the task can be restricted considerably by making a series of studies spaced over the whole range.

As a first example the activity of urease was investigated. The reason for this was that Quastel [1933] has recently found a number of substances belonging to the class of polyphenols (quinol, catechol, pyrogallol, adrenaline, *etc.*) all of which exerted a strong inhibitory action on urease. The corresponding quinones were found to be more toxic than the phenols. The inhibition could be counteracted by hydrogen sulphide and other thiol compounds. Quastel was of opinion that only the quinone actually inhibited the enzyme, and that such inhibition as was exhibited by the phenol was actually due to the presence of traces of quinone.

Since however these substances all form fairly strongly positive reversible oxidation-reduction systems and since other oxidising agents such as hydrogen peroxide are actually inhibitors it seems reasonable to suspect a relationship between the activity of the enzyme and the oxidation-reduction potential of the system.

In the present investigation the hydrolysis of urea by urease was studied in the presence of different types of reversible oxidation-reduction systems. In particular, it was important to study that part of the oxidation-reduction scale which is poised by the system quinol-quinone, that is to say the positive section. In addition a study was made of conditions at the negative end of the oxidation-reduction scale. The experimental difficulty of carrying out investigations with the very oxygen-sensitive negative systems, for example rosindulin or neutral red, is very great. Therefore the action of the enzyme was studied in a stream of hydrogen in the presence of an active catalyst in the form of palladised asbestos. Also, in the studies of the readily autoxidisable leucomethylene blue a stream of hydrogen was maintained through the apparatus while the hydrolysis was in progress. Other experiments were carried out in which a stream of air was employed and in which the reaction mixture was kept in a sealed vessel. The degree of hydrolysis of the urea was measured either by estimating the ammonia by aeration in the usual manner or by direct titration as follows: to a sample of the test solution an excess of standard trichloroacetic acid was added; the mixture was made up to a certain volume and filtered, and an aliquot part of the filtrate was titrated with $N/10$ NaOH to p_H 4·4 methyl orange being used as indicator. At this point the phosphoric acid used as a buffer had its first neutralisation point and consequently a minimum of buffering power. Free trichloroacetic acid being a strong acid can be titrated with this indicator without difficulty whereas ammonium salts do not give the acid colour.

The hydrogen employed was prepared in a Kipp's apparatus and purified by washing with potassium permanganate, mercuric chloride and alkaline pyrogallol. Air was purified by washing with acid in order to remove traces of ammonia. In all experiments in which a stream of gas was employed the outlet tube of the apparatus led into a vessel containing standard acid which was titrated for the estimation of the ammonia liberated. The enzyme preparation employed was either freshly ground soya bean or a 10 % aqueous extract thereof. The urea was purified by crystallisation. The palladised asbestos was prepared by carefully treating a warm suspension of finely divided asbestos in a very dilute solution of palladium chloride with glucose and sodium hydroxide.

Influence of air and hydrogen.

It was necessary to investigate whether any alteration of activity of urease could be detected in experiments in which air or hydrogen was bubbled through the solution, since Edlbacher *et al.* [1932; 1933, 1, 2] has detected a difference in the case of arginase. Similar reaction vessels containing 10 ml. of 6 % urea solution; 5 ml. M phosphate buffer p_H 7·4; 10 ml. 10 % aqueous soya bean extract; and 25 ml. distilled water, were set up. One was aerated at room temperature with air, whilst through another a stream of hydrogen was passed for the same time. In both cases the gas streams were regulated so that approximately one bubble per second passed through the solution, this being done in order to prevent frothing which might induce inactivation of the enzyme.

The following table gives the values of ammonia formed by fermentation in various experiments with different enzyme preparations (in ml. $N/10$ NH_4OH).

Exp. no.	Time min.	Closed vessel	Air stream	Hydrogen stream
1	85	4·65, 4·80	4·50, 4·65	—
2	205	9·35, 9·50	9·15, 9·05	—
3	95	4·30	—	4·40
4	220	9·15	—	9·05
5	95	—	9·80	10·00
6	40	—	5·20	5·40

This shows no significant difference between the tests.

Influence of active hydrogen.

In this experiment the reaction vessel contained 0·05 g. of palladised asbestos suspended with the enzyme preparation in the buffered medium. Hydrogen was passed through the mixture for 15 minutes after which the urea was added. In this manner it was ensured that the potential of the reaction mixture would be adjusted to the negative level of the hydrogen electrode at the p_H employed. At a temperature of 18° this is −427 mv. (referred to normal hydrogen electrode). The ammonia production was compared with that of two controls in closed vessels, one of which contained the same amount of catalyst.

The results of several experiments are:

Exp. no.	Time (min.)	Catalyst In hydrogen stream	Catalyst Closed vessel	Closed vessel
		Titrations in ml. $N/10$ NH_4OH		
7	85	—	7·45	7·75
8	205	—	13·70	15·30
9	85	—	7·05	7·45
10	205	—	13·95	14·70
1	85	4·45, 4·40	—	4·65, 4·80
2	205	8·85, 8·60	—	9·35, 9·50

This indicates a slight inhibition of the enzyme in the presence of the catalyst employed.

Influence of methylene blue.

Quastel [1932] has shown that methylene blue does not affect unpurified preparations of urease but that it exerts a strongly inhibiting action on the purified enzyme. Reaction vessels were set up, each containing 2 ml. 6 % urea solution, 30 ml. $M/5$ phosphate buffer p_H 7·4, 10 ml. 10 % soya bean meal extract. To one were added 10 ml. of 0·15 % methylene blue in $M/50$ phosphate buffer, to another the same solution reduced by hydrogen in the presence of palladised asbestos. The former experiment was aerated, whilst in the latter case a stream of hydrogen was employed. To a control 10 ml. of the buffer only were added. Hydrolysis was allowed to occur at room temperature.

Results of the ammonia estimations with different enzyme preparations in various times are given in the following table:

Exp. no.	Time min.	Methylene blue oxidised	Methylene blue reduced	Control
		Titrations in ml. $N/10$ NH_4OH		
11	90	17·2	16·9	—
12	80	4·55, 4·60	—	4·70, 4·60
13	120	7·30	—	7·30
14	60	13·70	—	13·10

If it be assumed that approximately 10 % of the methylene blue in the reduced preparation was present in the oxidised form the potential of this preparation can be regarded as −28 mv. (referred to the normal hydrogen electrode); that of the oxidised preparation (99 % oxidised) may be taken as

+58 mv. (The normal potential of the system methylene blue-leucomethylene blue at p_H 7·4 is zero.) It may be concluded, therefore, that the activity of the urease preparation employed is unaffected by potentials within the range −28 to +58 mv.

Ferro-ferricyanide system.

This system possesses a normal potential of approximately +450 mv. which is unaffected by hydrogen ion concentration. In the first place both extremes of the potential range were investigated. The potentials are not very sensitive to oxygen so that they can be measured electrometrically in the presence of air. As the substances are quite stable the hydrolyses were carried out in closed vessels kept in an incubator at 37°. Each vessel contained in a total vol. of 50 ml. 10 ml. 6 % urea solution; 5 ml. *M* phosphate buffer p_H 7·4; 5 ml. 10 % soya bean meal extract and 1 millimol of either ferro- or ferricyanide. Controls without addition were set up.

The respective ammonia productions in various experiments were:

Exp. no.	Time min.	Ferro-cyanide	Ferri-cyanide	Controls without addition
		Titrations in ml. *N*/10 NH_4OH		
7	85	7·70	7·15	7·75
8	205	15·20	13·90	15·30
9	85	7·45	6·80	7·45
10	205	14·75	13·65	14·70

The oxidation-reduction potentials were measured with gilded platinum electrodes against a standard calomel electrode. Owing to inadequate poising the readings were not quite steady; the values for ferrocyanide ranging between −22 and +10 mv., those for ferricyanide between +264 and +336 mv. Referred to the normal hydrogen electrode these values become +228 to +260 mv. (ferrocyanide) and +514 to +586 mv. (ferricyanide). The rate of hydrolysis of urea was unaffected by these substances, apart from a very slight inhibition exerted by the ferricyanide.

Influence of quinol.

The poisoning of urease by quinol which was observed by Quastel, and which can be demonstrated both with the crude soya bean meal and with extracts, can, as he also showed, be prevented by the addition of substances containing the SH group. The inhibition thus removed could be prevented likewise by treatment with active hydrogen. Three experiments were set up. In each the reaction vessel contained in a total volume of 50 ml. 10 ml. 6 % urea solution; 5 ml. *M* phosphate buffer solution p_H 7·4; 10 ml. of a 10 % aqueous soya bean meal extract or the corresponding quantity of soya bean meal. To the contents of two vessels 0·1 g. quinol was added, the third serving as a control. One of the vessels containing the quinol was exposed to a stream of hydrogen in contact with 0·05 g. palladised asbestos for 15 minutes before the enzyme preparation was added, and for a further 15 minutes afterwards. The urea solution was then added and the stream of hydrogen maintained during the hydrolysis.

Results of ammonia estimations in various experiments are shown in the following table:

Exp. no.	Time min.	No. 1 quinol Pd-catalyst in hydrogen stream	No. 2 quinol (closed vessel)	No. 3 control	
		Titrations in ml. *N*/10 NH_4OH			
15	125	4·30	0	4·55	soya bean extract meal
16	210	8·80	0	9·15	
17	120	6·50	0·90	7·05	

The oxidation-reduction potential in No. 2 was between −53 and −34 mv. measured against the standard calomel electrode (*i.e.* +197 to +216 mv., referred to the normal hydrogen electrode).

It is apparent therefore that there is almost complete inhibition of urease in the presence of quinol and that this is prevented by reduction with hydrogen in the presence of catalytic palladium.

Discussion.

The activity of urease was found to be unaffected by the oxidation-reduction potential of the medium within the range investigated—namely, that covered by the systems palladium-hydrogen, methylene blue-leucomethylene blue, and ferri-ferrocyanide. Inhibition by quinol is apparently a specific action which is not related to the oxidation-reduction potential. It can probably be ascribed, as Quastel has suggested, to the action of traces of quinone rather than to the quinol itself, because no inhibition occurs when a stream of hydrogen is passed in the presence of palladium. It can be concluded that the reaction of the enzyme with quinone, if it is oxidative in nature, is not reversible in the thermodynamic sense; otherwise ferricyanide with a potential of more than +500 mv. would exert a stronger action than that of quinol with only +200 mv.

Summary.

1. A stream of hydrogen or air did not affect the activity of urease. Moreover, activation of the hydrogen by palladium-asbestos had no appreciable influence on the rate of hydrolysis of urea.

2. The reversible oxidation-reduction systems, methylene blue and ferro-ferricyanide, did not influence the activity of the enzyme.

3. Quinol inhibits urease action, but on reduction with active hydrogen this effect disappears. It is believed that the original inhibition is due to a chemical reaction with the enzyme rather than to an influence on the oxidation-reduction potential.

The author wishes to express his thanks to Prof. J. B. S. Haldane and Prof. J. C. Drummond for giving the facilities of carrying out this work and for much valuable criticism and advice.

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LVII. DEHYDROGENASES OF THE FILTRABLE ORGANISM OF *AGALACTIA*.

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The detection of dehydrogenases in *Agalactia* has been reported in a previous paper [Pirie and Holmes, 1933] where the great instability of the enzymes has also been demonstrated. The experiments reported in this paper were undertaken in order to see how many substrates the organism could oxidise. *Agalactia* and *Pleuropneumonia* are the smallest organisms so far shown to possess enzymes, and it seemed possible that the number of enzymes might be very restricted and their kinetics rather different from those of ordinary bacterial enzymes. For the sake of simplicity *Agalactia* will be described as a virus in this paper in spite of the paper by Ledingham [1933] in which many different forms of the organism are described. This pleomorphism does not entirely differentiate *Agalactia* from viruses since Bedson [1933] has described a life cycle for the virus of psittacosis which involves many different and some large forms. While admitting that the exact classification of *Agalactia* is still in doubt the fact of its filtrability enables it to be considered as a very small organism.

The experimental procedure has been described fully in the previous paper. *Agalactia* has been grown on Hartley's broth *plus* 5–10 % horse-serum at 37° for 3 days. In order to obtain the virus free from the medium the culture was centrifuged at 3500 r.p.m. for one hour and the sediment was drained and rubbed up in saline. The volume of saline was usually 1/10th that of the original culture. The enzymes of this suspension could then be investigated. The oxygen uptake and the methylene blue reduction times in the presence of different substrates were measured. In every experiment a saline control and also a control of the virus *plus* some substrate which was known already to be oxidised were included since occasionally apparently well-grown cultures failed to show any dehydrogenases whatever. Table I shows the results obtained using the methylene blue technique. These indicate that a considerable number of substrates are oxidised by *Agalactia* if the substrate concentration is high enough. *Agalactia* oxidises very few compounds if their final concentration is less than $M/10$. Lactate, adenylic acid, adenosine, fructose and hexosediphosphate are the only substrates which are oxidised in low concentration. In this respect *Agalactia* resembles *Esch. coli* since Quastel and Whetham [1925] found that the dehydrogenases of the resting bacterium varied enormously in their affinity for their substrate and relatively few enzymes attacked substrates in low concentrations. The significance of enzymes which do not attack a substrate unless it is in high concentration is obscure. It seems possible that it is a question of incomplete specificity of enzymes so that each dehydrogenase has more than one substrate but has enormously varying affinities for the different substrates which it may attack. Table II shows the effect of variation of substrate concentration on the velocity of reduction of methylene blue.

Table I. *Dehydrogenases of* Agalactia.

Each Thunberg tube contained 1·0 ml. virus suspension, 1·0 ml. substrate, 1·0 ml. *M*/10 phosphate buffer of p_H 7·4 and 0·2 ml. 1/3000 methylene blue. The final substrate concentrations are given.

Substrate	Red. time in mins.	Substrate	Red. time in mins.
Broth	11, 6	Formate *M*/6	60
Serum	10	Succinate *M*/6	39
Lactate *M*/20	7, 11	Malate *M*/6	12
Glucose *M*/6	31	Oxalate *M*/6	∞
Fructose *M*/6	14	Gluconate *M*/6	∞
Galactose *M*/6	18	Glutamate *M*/12	56
Lactose *M*/6	24	Aspartate *M*/12	40
Adenylic acid *M*/300	17	Alanine *M*/6	48
Adenosine *M*/300	24	Citrate *M*/6	42
Adenine (saturated)	∞	Hexosediphosphate *M*/300	25
Xanthine	∞	Hexosemonophosphate	∞
Saline	∞		

Table II. *Effect of variation of substrate concentration on the reduction time of methylene blue.*

Conditions the same as in Table I.

Substrate	Conc.	Red. time	Substrate	Conc.	Red. time
Glucose	1/3 saturated	30	Lactose	*M*/6	38
	M/6	32		*M*/30	∞
	M/30	∞	Galactose	*M*/6	39
Fructose	*M*/3	14		*M*/30	60
	M/30	28		*M*/100	∞
	M/300	23	Succinate	*M*/6	46
Lactate	*M*/30	24		*M*/30	∞
	M/300	20	Aspartate	*M*/12	43
	M/1500	85		*M*/60	120

Alternatively it suggests that a co-enzyme is necessary for the reaction or that an intermediate compound, not the actual substrate added, is the true substrate for the enzyme. The effect of the addition of the co-enzyme for liver glucose dehydrogenase, described by Mann [1932], on the velocity of glucose oxidation by *Agalactia* was investigated. It was found that the co-enzyme preparation itself was rapidly oxidised and that no acceleration of glucose oxidation could be shown. The reduction time of a mixture of glucose and co-enzyme was approximately the mean of the reduction times for the separate substrates as though only one enzyme were concerned. The same is true for mixtures of fructose or galactose with the co-enzyme or with adenylic acid. Since *Agalactia* dehydrogenates adenosine but has no effect on adenine it is presumably the carbohydrate part of the molecule that is attacked. There seems to be only one enzyme in *Agalactia* which oxidises all the different carbohydrates since a mixture of any two of these is oxidised at the mean rate of oxidation of the two separately. It is possible that the co-enzyme of glucose dehydrogenase also contains a carbohydrate radical since it is oxidised by the same enzyme (Table II).

The addition of phosphate buffer to the mixture of virus and substrate in the vacuum tube often greatly decreased the reduction time. This was at first thought to be a specific effect of phosphate since borate and sulphate had no accelerating effect. Quastel and Whetham [1924] state that replacement of phosphate by borate had no effect on the activity of succinic dehydrogenase of *Esch. coli*. But it seems more probable that the accelerating action of phosphate

Table III. *Unity of carbohydrate dehydrogenases of* Agalactia.

Final conc. of total sugars $M/4$. Reduction times in minutes.

		Calc. mean			Calc. mean			Calc. mean
Glucose	31	—	Fructose	14	—	Lactose	24	—
+Fructose	16	22	+Glucose	16	22	+Glucose	24	27
+Lactose	24	27	+Lactose	16	19	+Fructose	16	19
+Galactose	21	24	+Galactose	14	16	+Galactose	21	21
Galactose	18	—	Fructose	33	—	Glucose	32	—
+Fructose	14	16	+Co-enzyme	28	28	+Co-enzyme	26	27
+Glucose	21	24	Fructose	43	—	Glucose	32	—
+Lactose	21	21	+Adenylic acid	24	32	+Adenylic acid	25	27

on the enzymes of *Agalactia* is a p_H effect, since some of the dehydrogenases have a sharp optimum p_H of 7·4. In vacuum tubes containing no buffer the p_H rises during evacuation and therefore the enzyme may not be acting under optimum conditions. It was impossible to be certain of this point since experiments with citrate and glycylglycine as buffers were inconclusive. Those enzymes which seemed most affected by phosphate were also the most unstable and were often destroyed after the suspension of *Agalactia* had stood on the bench for half an hour. In order to test the relative effects of different buffers all vacuum tubes had to be evacuated simultaneously. Since it seemed possible that phosphate had either a stabilising or stimulating action on some of the enzymes of *Agalactia* it was thought that it might have a similar action on the organism itself. The sediments from equal volumes of culture were suspended in equal volumes of $M/10$ phosphate buffer of p_H 7·4 or in physiological saline of the same p_H. The suspensions were kept on the bench and the activity of some dehydrogenases was determined at intervals. It was found that the glucose and succinic dehydrogenases were destroyed more rapidly in phosphate than in saline whereas the lactic dehydrogenase was equally stable in either solution. Elford and Galloway [1933] found that the virus of louping-ill was less stable, as judged by its pathogenicity, in phosphate solutions than in saline and it would be tempting to consider that this case also is one of enzyme destruction.

The dehydrogenases of *Agalactia* varied considerably in their relative activities. Many cultures were investigated under standard conditions before this variability was accepted as being a real occurrence not due to chance changes in technique. The ratio of the glucose and lactic dehydrogenases was particularly investigated. First of all the ratio of the two enzymes at different stages of growth of the culture was estimated. 30 ml. samples were removed from cultures on the 2nd, 3rd and 4th days of growth and centrifuged for one hour, and the lactate and glucose dehydrogenase activity was estimated in the usual way. A saline control was also put up, the virus sediment being rubbed up in 3·0 ml. saline. If an active glucose dehydrogenase was present the ratio of the enzymes was usually 2–3 to 1 at all stages of growth, but if a very feeble one only was detectable this seemed to be at its maximum on the 3rd day of growth when the ratio might be 7:1.

The variability depended more noticeably on the medium. Different batches of Hartley's broth gave very different glucose dehydrogenases although the lactic dehydrogenase was constant. In order to see if the medium were the controlling factor in variability of the enzymes a culture grown on a broth which tended to diminish the glucose enzyme was transplanted to a "good"

broth. The enzyme activity increased considerably. After two cultures on this broth a subculture was made on to the "bad" broth and the enzymes were estimated again. There was a tendency for the glucose and other dehydrogenases to disappear in the first subculture, the lactic dehydrogenase being the only one left. After several generations the enzymes might return even on the bad medium. Variability therefore seems to be neither completely a matter of medium nor a matter of phase of growth. It is possible that different media

Table IV. *Variability of enzymes.*

(*a*) Comparison of enzymes on two broths.

	Type of broth		
Substrate	"Bad"	"Good"	"Bad"
Control	∞	101	∞
Glucose *M*/1	∞	48	∞
Lactate *M*/10	14	28	12
Formate *M*/2	182	39	∞
Fructose *M*/10	180	—	—

(*b*) Glucose and lactate dehydrogenases of successive cultures on different broths.

	Type of broth			
Substrate	"Good"	"Bad"	"Bad"	"Bad"
Saline	200	28	101	138
Glucose *M*/1	36	28	100	70
Lactate *M*/10	26	6	8	10

Conditions are the same as in previous tables. Reduction times are given in minutes.

influence the preponderance of one form of the organism over any other [Ledingham, 1933]. Microscopical preparations of the cultures by Ledingham's method for the detection of the different forms was not carried out, but bacterial sterility was tested by inoculation into broth and by Gram staining of the sediment from the centrifuged culture.

A more detailed study of the lactic dehydrogenase of *Agalactia* was made since this enzyme seemed to be the most active and stable one possessed by the virus. Quastel found that the lactic dehydrogenase of *Esch. coli* was inhibited by other acids containing the groups —CHOH.COOH or —CO.COOH. The effect of such acids on the activity of the lactic enzyme of *Agalactia* was investigated in order to see if this was of the same type as the enzyme contained in *Esch. coli*. The solution of the inhibitor was added to the virus before the lactate in order that it might be adsorbed at the surface non-competitively. The time taken by the virus to reduce methylene blue in the presence of lactate, the inhibiting acid and a mixture of the two was then determined. Solutions of oxalate and gluconate inhibited lactic dehydrogenase almost completely while glycollic and glyceric acids had slight inhibitory actions. Glucose, citrate, tartrate and malate had no effect. Gluconate or oxalate was added to the medium in which *Agalactia* was grown in order to see if any depressing action on the growth of the organism could be observed. Lactic dehydrogenase was so much the most active of the enzymes so far detected in *Agalactia* that it seemed possible that if this enzyme were inhibited the growth of the organism might be impaired. It was found that the addition of 1·0 % oxalate to the medium entirely prevented growth of *Agalactia*. Oxalate in lower concentrations permitted some growth, while concentrations of gluconate up to and including 1·0 % retarded but did not inhibit the growth of *Agalactia*. Subcultivation on

to normal medium gave normal growth. The addition of broth had a slight inhibitory effect on lactic dehydrogenase showing that under normal conditions of culture the enzyme cannot be optimally active. It seems unlikely that the concentration of any inhibitor occurring naturally will be great enough to inhibit growth.

The oxygen uptake of *Agalactia* in the presence of different substrates was measured in Barcroft differential manometers. Lactate, broth, serum, fructose and citrate were regularly oxidised while aspartate, alanine and succinate were irregularly attacked. In general it seemed that those substances which were dehydrogenated regularly in the presence of methylene blue could also be oxidised by oxygen while those which were only occasionally hydrogen donators to methylene blue were also only occasionally oxidised in air. The oxygen uptake of *Agalactia* in presence of broth was linear for the whole course of the experiment while the uptake in the presence of all other substrates fell off usually during the second hour. It was found that in presence of broth the enzymes remained active, while without it they became inactive during the shaking. The destruction of the enzymes was determined by testing the reducing capacity of the organism in the presence of fresh broth or lactate at the end of the experiment. *Agalactia* which has been shaken in the manometer with broth still decolorises methylene blue rapidly whereas after shaking in the presence of any other substrate the reduction of methylene blue does not occur. This protective effect of broth is unexplained.

Summary.

A more comprehensive survey of the dehydrogenases and oxidising enzymes of *Agalactia* has been made. The most active dehydrogenases are those attacking lactate, adenylic acid, adenosine, fructose and hexosediphosphate. Other dehydrogenases need very high substrate concentrations. The relative strengths of the dehydrogenases vary from culture to culture. This variability does not seem to depend entirely on the medium or on the phase of growth at which the enzymes are estimated. The lactate enzyme is similar to the lactic dehydrogenase of *Esch. coli* in that it is strongly inhibited by gluconate and oxalate.

I have much pleasure in thanking the Medical Research Council for a personal grant and Mrs B. E. Holmes for assistance in this work. Also I wish to thank Mr P. J. G. Mann for some glucose dehydrogenase co-enzyme.

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LVIII. A FURTHER NOTE ON THE CHLORIDES AND INORGANIC CONSTITUENTS OF THE SERUM AND CEREBROSPINAL FLUID IN NEPHRITIS.

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Linder and Carmichael [1931] recorded the results of a study of the inorganic constituents of the serum and cerebrospinal fluid in four cases of advanced nephritis. They hoped to find an explanation for the very high level of the cerebrospinal fluid-chloride which has been found in some cases of uraemia. But cases of uraemia showing this feature are somewhat rare, for the highest they found over a period of some years was 761 mg./100 ml. This was in Case IV of the above paper, and the findings in that case are included in Table I, together with those in Hamilton's [1925] case Mc. Gn. which is the only other case of this kind for which data are available. In both of these the relationship of the chloride of the cerebrospinal fluid to the chloride of the serum and also that of the bicarbonate showed unusual departures from the normal. It was therefore suggested that a breakdown of the normal regulating mechanism was the condition which gave rise to the occasional very high chlorides in uraemia.

The following figures (Case V) were obtained from the arterial blood and cerebrospinal fluid of a man suspected of having meningitis. The discovery that the cerebrospinal fluid-chloride was 850 mg./100 ml. led to the diagnosis of uraemia. Even at rest dyspnoea was intense. Death occurred 12 hours later.

All methods employed were the same as those used by Linder and Carmichael [1931].

Table I.

	Blood-urea mg./100 ml.	Chloride: Serum-water mM	Chloride: CSF mM	Cl_{SW}/Cl_{CSF}	Bicarbonate: Serum-water mM	Bicarbonate: CSF mM	$HCO_{3\,SW}/HCO_{3\,CSF}$	Total base: Serum-water mM	Total base: CSF mM	B_{CSF}/B_{SW}	Inorganic phosphorus: Serum-water mM	Inorganic phosphorus: CSF mM	Protein: Serum g./100 ml.	Protein: CSF g./100 ml.	Other acids: Serum-water mM	Other acids: CSF mM
Mc. Gn.	—	118	142	0·83	16	16	1·0	178	174	0·98	—	1·3	6·8	—	—	—
Case IV	380	98	127	0·77	13·0	13·2	0·98	164	—	—	4·7	5	7·0	0·09	26	—
Case V	800	116	145	0·80	2·8	7·4	0·38	167·5	154·5	0·92	2·5	0·9	7·3	0·175	31	2

The points of interest in the results from Case V are (1) that the serum-water chloride has not risen in proportion to the cerebrospinal fluid-chloride, and therefore the ratio Cl_{SW}/Cl_{CSF} is 0·80; this is lower than any recorded ratio except that of Case IV; (2) that the acidosis is extreme, the bicarbonate being so low that the formula suggested by Hamilton correlating serum-water and cerebrospinal fluid-bicarbonates could not be applied; (3) that the total base

shows a normal distribution; (4) that the distribution of phosphorus and undetermined anions suggests that the meningeal barrier is able to resist the passage of these into the cerebrospinal fluid.

In Case IV the acidosis was less severe, but the normal regulating mechanism showed greater disturbance, for the bicarbonate was the same in the two fluids instead of being higher in the cerebrospinal fluid as Hamilton's formula would require; the phosphorus was the same instead of being lower in the cerebrospinal fluid, and the chloride ratio was reduced to 0·77. The conditions found by Hamilton in Mc. Gn. were similar to those in Case IV.

The hypothesis that the very high chloride in the cerebrospinal fluid is concerned with a departure from the normal relationship to the chloride of the serum is supported by these additional results. But it is evident that a second important factor is the level of the serum-water chloride. When this is high as in Case V and in Mc. Gn. the cerebrospinal fluid chlorides reach a higher level than in Case IV, although the disturbance of chloride equilibrium was greater in the last. A third factor may possibly be the retention by the meningeal barrier of the power to resist the invasion of the cerebrospinal fluid by phosphates, sulphates and other anions from the serum, thus preserving base to carry chloride and bicarbonate. Atchley and Benedict [1930] found that a decrease in serum-chlorides was the rule in uraemia, but Bulger *et al.* [1926] point out that in uraemia with high serum-chlorides the bicarbonate may be extremely low, which was so in Case V. These facts explain the rarity of very high cerebrospinal fluid-chlorides in uraemia and their evil significance.

SUMMARY.

A very high cerebrospinal fluid-chloride titre in uraemia indicates the presence of (i) a disturbance of the normal serum-cerebrospinal fluid relationships, and (ii) a high serum-chloride with a very severe acidosis.

My thanks are due to Dr J. F. Wicht, Medical Superintendent of the City Hospital, Capetown, for the opportunity to study this patient who was under his care, and to Prof. B. J. Ryrie for his interest in this investigation.

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LIX. THE EFFECT OF HALOGEN SALTS ON STEAPTIC DIGESTION.

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(*Received February 2nd, 1934.*)

Following on work as to the effect of halogen salts on salivary, peptic and tryptic digestion [Clifford, 1925; 1927; 1928; 1933], it was decided to carry out experiments on their action on lipase.

The effect of halogen salts on lipolytic activity has been investigated many times with varying results. Loevenhart and Pierce [1907], using pancreatic and hepatic lipases, found sodium fluoride strongly inhibitory, as also, though to a far less degree, were sodium chloride and iodide, potassium chloride and iodide and potassium bromide. Sodium bromide, however, was found by these observers to exert a slight accelerating action. Amberg and Loevenhart [1908] confirmed the inhibitory action of sodium fluoride on hydrolysis by hepatic lipase.

Mellanby and Woolley [1914] found no effect on lipolytic activity with the chlorides of sodium, calcium and magnesium, but Falk [1918] stated that sodium chloride accelerated the action over a period of 30 minutes. This acceleration disappeared if the fat were previously emulsified and was replaced by a retardation if the concentration of chloride were increased.

Leake *et al.* [1929] found that sodium fluoride in a concentration of 1 : 50,000 inhibited hepatic lipase *in vitro* and extended the work to observations *in vivo*, obtaining similar results. In the same year Corran [1929] found the chlorides of sodium and potassium to be slightly augmentative, and calcium chloride without effect. McGillivray [1930] confirmed these observations for sodium chloride but stated that calcium chloride also hastened lipase action.

These results, except for agreement as to the inhibitory action of sodium fluoride, vary considerably, and it was decided to investigate the action of various concentrations of halogen salts on the rate of lipolytic activity.

Experimental methods.

Since in the digestion of fats emulsification is obviously of great importance, the substrate used was the relatively stable emulsion found naturally in cow's milk.

A solution of commercial pancreas substance was used as the source of lipase, since in a low concentration it showed marked lipolytic activity together with a negligible tryptic action. A solution of the pancreas substance in the concentration used in these experiments showed no proteolytic action (biuret reaction unchanged in each case and this negative result confirmed by Sørensen titrations with fish-protein) in 5 hours, when incubated together with sodium carbonate at 37° with caseinogen, fish-protein or hard-boiled egg-white.

In any set of experiments 200 ml. of milk were placed in a flask together with the requisite weighed amount of halogen salt. The flasks were allowed to reach a

temperature of 37° in a water-bath, when 20 ml. of a 0·5 % solution of pancreas substance also warmed to 37° were added; 25 ml. of this mixture were removed immediately, about 150 ml. of boiling water added to stop enzyme action, and the liquid was titrated against 0·1 *N* NaOH using phenolphthalein as an indicator.

Further 25 ml. portions were removed at 15 minute intervals and treated similarly.

The milk used had been pasteurised and gave a negligible rise in acidity when kept for 2 hours at 37°.

A typical experimental result is as follows:

200 ml. milk with 0·125 *M* LiBr.

20 ml. 0·5 % pancreas substance.

25 ml. removed at 15 minute intervals and titrated with 0·1 *N* NaOH.

Minutes	ml. 0·1 *N* NaOH	ml. 0·1 *N* acid produced
0	3·6	—
15	6·8	3·2
30	8·9	5·3
45	11·7	8·1
60	13·6	10·0
75	13·8	10·2
90	14·7	11·1
105	14·5	11·2

In each series two control flasks were used and one of each of the halogen mixtures. The concentrations investigated varied from 0·5 to 0·008 *M*. Three separate batches of pancreas substance were used and at least a similar number of samples of each halogen salt.

Results.

Fluorides. Typical results with fluorides are given in Table I. From them it can be seen that sodium, potassium and ammonium fluorides are strongly

Table I. *Fluorides.*

ml. 0·1 *N* acid produced.

Time of digestion in minutes	15	30	45	60	75	90	105	15	30	45	60	75	90	105
				0·5 *M*							0·25 *M*			
Control A	0·3	2·4	4·6	6·2	7·2	8·3	8·9	0·9	2·1	4·0	5·0	6·0	6·6	7·4
,, B	0·8	3·0	5·0	6·4	7·4	8·9	9·1	0·5	2·4	3·8	4·8	5·6	7·0	7·1
Na	0	0	0·7	1·5	1·9	2·1	2·3	0	0	0·1	0·3	0·5	0·5	0·6
K	0	0	0	0·4	1·0	1·5	2·1	0·1	0·4	1·0	1·2	1·7	1·7	2·1
NH_4	0	0	0	0·1	0·5	1·6	2·1	0	0	0·1	0·1	0·1	0·2	0·7
				0·125 *M*							0·06 *M*			
Control A	2·1	4·6	6·4	7·9	9·1	10·7	11·0	2·1	4·8	6·4	8·2	8·9	9·7	10·6
,, B	2·0	4·4	6·3	7·8	8·7	10·5	10·9	2·5	4·7	6·3	8·0	8·7	10·2	10·4
Na	1·0	2·6	3·6	4·2	4·4	4·9	5·7	1·3	2·5	3·5	4·8	5·7	6·3	7·6
K	0·8	2·5	3·6	3·8	4·3	4·5	5·5	0·9	2·7	3·2	3·8	4·8	5·6	6·0
NH_4	0·6	0·8	0·9	1·4	1·4	1·6	2·4	0·2	0·4	1·3	2·5	3·7	4·5	4·6
				0·03 *M*							0·015 *M*			
Control A	2·9	4·8	7·6	9·0	9·9	10·3	11·2	2·2	5·3	6·9	8·6	9·2	10·2	11·3
,, B	3·0	5·3	7·2	8·8	9·9	10·8	11·0	2·5	5·2	7·1	8·5	9·5	9·9	11·2
Na	2·1	4·0	5·0	6·4	7·1	7·4	8·7	1·7	4·5	5·4	6·2	7·3	8·9	9·0
K	2·2	3·9	5·7	7·7	7·9	8·6	8·9	1·7	4·4	5·7	7·0	7·5	7·8	9·0
NH_4	2·3	4·2	5·8	6·8	7·6	8·6	8·9	1·1	4·2	5·8	6·9	7·6	8·1	9·2
				0·008 *M*										
Control A	0·3	2·4	4·3	4·5	5·9	6·9	8·9							
,, B	2·4	3·0	4·1	4·4	5·6	6·8	8·3							
Na	1·1	1·8	2·6	3·2	3·6	5·0	6·5							
K	0·6	1·2	2·4	2·6	3·5	4·4	5·4							
NH_4	1·4	1·8	3·2	3·8	4·2	4·7	5·9							

Table II. *Chlorides.*

ml. 0·1 *N* acid produced.

Time of digestion in minutes	15	30	45	60	75	90	105	15	30	45	60	75	90	105
				0·5 *M*							0·25 *M*			
Control A	1·2	3·3	6·3	8·4	8·7	9·6	10·0	2·3	4·5	6·2	7·1	9·1	9·7	10·6
" B	1·4	4·5	5·9	8·3	8·9	9·5	10·1	2·0	4·4	5·9	7·0	9·2	9·9	10·4
Li "	1·2	4·8	6·0	7·8	9·0	9·9	10·3	1·6	4·4	6·0	7·9	9·1	9·4	9·9
Na	0·9	4·5	6·6	8·0	8·3	8·9	9·5	1·4	4·6	5·8	7·1	9·3	9·5	10·0
K	0·8	4·7	5·8	8·2	8·8	9·2	9·7	1·7	3·8	5·7	7·9	9·0	9·9	10·0
NH_4	0·8	3·6	6·3	7·5	8·6	9·0	9·6	1·8	4·5	5·2	7·3	9·7	10·0	10·4
				0·125 *M*							0·06 *M*			
Control A	2·1	4·9	7·1	8·8	10·6	11·1	12·0	1·7	3·4	5·6	7·7	8·5	9·7	10·4
" B	2·3	4·4	6·2	7·8	10·8	10·8	11·4	1·5	3·0	5·3	7·4	8·3	9·1	10·7
Li "	2·1	4·5	6·7	7·3	9·6	10·2	12·1	1·8	2·9	5·5	7·5	8·0	8·9	10·2
Na	1·9	5·0	7·6	8·6	10·3	11·4	11·5	1·5	3·1	5·4	7·6	8·4	9·3	10·5
K	1·4	4·7	6·4	7·9	10·0	10·7	12·0	1·6	3·5	5·2	7·7	8·2	9·5	10·6
NH_4	1·8	4·2	6·1	7·3	9·4	11·3	11·3	1·8	3·3	5·5	7·6	8·4	9·4	10·3
				0·03 *M*							0·015 *M*			
Control A	2·5	4·7	6·3	8·0	8·7	10·2	10·4	3·3	6·6	8·4	9·5	11·9	12·2	12·3
" B	2·0	4·5	6·1	8·2	8·9	10·3	10·6	3·0	6·4	8·5	9·8	11·7	12·0	12·5
Li "	1·9	4·0	5·9	8·0	8·7	10·4	10·7	3·1	6·3	8·4	9·6	11·6	11·9	12·2
Na	2·3	4·4	6·0	8·4	8·8	10·1	10·5	3·3	6·5	8·5	9·7	11·5	12·1	12·4
K	2·4	4·3	6·2	7·9	8·5	10·4	10·6	3·1	6·2	8·6	9·4	11·3	12·2	12·4
NH_4	2·2	4·4	6·4	8·0	8·6	9·9	10·3	3·2	6·6	8·5	9·5	11·5	12·3	12·5
				0·008 *M*										
Control A	1·9	3·4	6·5	7·7	9·5	10·7	11·6							
" B	2·0	3·5	6·7	7·9	9·8	10·8	11·9							
Li "	1·9	3·3	6·4	7·8	9·7	10·7	11·5							
Na	2·1	3·5	6·6	7·5	9·6	10·6	11·7							
K	2·3	3·6	6·6	7·7	9·8	10·8	11·5							
NH_4	2·0	3·4	6·5	7·6	9·7	10·7	11·8							

Table III. *Bromides.*

ml. 0·1 *N* acid produced.

Time of digestion in minutes	15	30	45	60	75	90	105	15	30	45	60	75	90	105
				0·5 *M*							0·25 *M*			
Control A	1·0	2·5	4·3	5·8	6·6	7·5	7·5	1·3	5·0	7·0	9·2	9·7	10·1	12·3
" B	1·0	2·6	4·3	6·3	6·6	7·2	7·6	1·1	4·9	6·8	9·0	9·5	9·9	11·8
Li "	0·3	1·9	3·9	4·3	5·1	5·7	6·1	0·7	4·5	7·2	8·5	9·2	9·4	11·2
Na	0·2	1·5	3·3	4·5	5·1	6·7	6·7	0·9	3·4	6·7	7·7	8·6	9·6	11·5
K	0·5	1·9	3·5	4·6	5·3	5·8	6·6	0·5	3·4	6·6	8·6	9·0	9·8	10·8
NH_4	0·3	1·1	3·7	5·0	5·2	5·6	6·5	3·1	4·3	7·7	8·4	8·6	9·4	11·7
				0·125 *M*							0·06 *M*			
Control A	2·2	5·3	6·9	8·6	9·2	10·2	11·3	1·4	3·4	6·5	8·6	8·9	9·9	10·2
" B	2·3	5·4	7·0	8·3	9·3	10·0	11·1	1·5	3·7	6·4	8·5	9·1	9·8	10·3
Li "	2·0	5·5	7·1	8·5	9·1	10·3	11·0	1·5	3·6	6·6	8·4	9·0	10·0	10·4
Na	2·1	5·4	7·0	8·4	9·4	10·2	11·2	1·3	3·5	6·4	8·5	8·9	10·1	10·3
K	2·3	5·6	7·2	8·5	9·2	10·1	11·3	1·2	3·4	6·3	8·6	9·2	9·9	10·3
NH_4	2·2	5·1	7·2	8·6	9·5	10·4	11·4	1·5	3·8	6·6	8·7	9·0	10·1	10·4
				0·03 *M*							0·015 *M*			
Control A	2·0	4·8	7·1	8·6	10·4	11·0	11·9	2·3	4·3	7·2	8·9	9·7	10·5	11·0
" B	2·1	4·0	6·0	7·6	10·7	11·0	11·5	2·0	4·7	7·0	8·9	9·9	10·4	10·9
Li "	2·0	4·3	6·5	7·1	10·3	10·9	11·8	2·1	4·3	7·5	9·1	9·8	10·6	10·8
Na	1·9	4·7	7·2	8·4	10·5	11·2	11·7	2·1	4·5	6·9	9·3	9·8	10·5	10·7
K	1·6	4·8	6·5	8·3	10·4	11·1	11·7	2·2	4·7	7·4	9·2	10·0	10·6	11·1
NH_4	2·1	4·6	6·5	7·7	10·6	11·5	11·6	1·9	4·4	7·1	8·9	9·7	10·5	10·9
				0·008 *M*										
Control A	1·7	2·9	5·0	7·5	9·4	10·0	10·5							
" B	1·3	2·8	5·0	7·4	9·2	9·8	10·1							
Li "	1·5	2·7	4·9	7·3	9·0	9·9	10·4							
Na	1·8	3·4	5·3	7·5	8·9	9·7	10·5							
K	1·4	2·9	5·1	7·6	9·0	9·8	10·3							
NH_4	1·5	2·8	5·3	7·5	9·4	9·9	10·4							

Table IV. *Iodides.*

Time of digestion in minutes	ml. 0·1 *N* acid produced.													
	15	30	45	60	75	90	105	15	30	45	60	75	90	105
	0·5 *M*							0·25 *M*						
Control A	3·1	5·7	7·9	8·5	9·7	10·4	10·7	1·3	2·4	4·6	6·2	7·2	8·3	8·9
B	3·2	5·3	7·8	8·4	9·2	9·9	10·0	1·8	3·0	5·0	6·4	7·4	8·9	9·4
Li "	2·7	5·2	7·3	7·6	9·0	9·4	10·4	0·9	2·5	4·7	6·0	7·1	8·5	9·3
Na	3·6	6·5	8·7	8·9	9·7	10·4	10·6	1·1	2·7	4·8	6·3	7·3	8·5	9·2
K	3·5	5·3	8·1	8·6	9·2	10·7	10·9	1·3	2·3	4·5	6·3	7·4	8·6	9·2
NH_4	3·1	5·5	8·3	8·5	9·7	10·7	11·2	1·2	2·6	4·9	6·4	7·5	8·9	9·4
	0·125 *M*							0·06 *M*						
Control A	0·8	2·0	3·1	4·9	5·2	6·1	6·9	2·1	4·4	6·2	7·7	9·0	10·6	10·9
B	0·7	2·1	3·4	4·8	5·9	6·5	7·1	2·0	4·2	6·1	7·9	8·9	10·5	11·0
Li "	1·5	2·8	4·2	5·5	5·7	6·7	7·0	1·9	4·0	6·3	8·0	9·1	10·7	11·2
Na	0·8	2·4	3·5	4·9	5·4	6·2	7·4	2·0	4·4	6·4	7·8	9·0	10·4	10·8
K	0·5	2·2	3·1	4·9	5·8	6·6	7·2	2·1	4·3	6·4	7·6	9·1	10·6	11·0
NH_4	0·6	2·5	3·2	4·7	5·6	6·5	7·1	1·9	4·2	6·5	7·8	8·9	10·6	11·1
	0·03 *M*							0·015 *M*						
Control A	2·5	4·4	7·5	9·0	9·7	10·5	11·2	2·9	5·2	7·5	8·0	9·2	10·1	10·7
B	2·3	4·7	7·3	8·9	9·5	10·2	10·7	3·0	5·3	7·8	8·2	9·0	9·9	10·4
Li "	2·3	4·5	7·9	9·1	9·9	10·4	11·0	2·8	5·2	7·4	8·3	9·3	10·2	10·6
Na	2·1	4·3	7·4	9·0	9·8	10·5	10·8	2·9	5·5	7·9	8·1	9·2	10·4	10·6
K	2·2	4·7	7·8	9·2	10·0	10·7	11·1	3·1	5·6	8·0	8·6	9·2	10·1	10·8
NH_4	2·0	4·4	7·8	9·5	9·9	10·5	10·9	2·9	5·5	7·8	8·5	9·1	10·4	10·9
	0·008 *M*													
Control A	2·0	5·0	7·1	8·5	10·4	10·9	11·5							
B	2·3	4·6	6·9	8·8	10·5	11·0	11·4							
Li "	2·1	4·7	6·7	8·3	10·5	10·8	11·6							
Na	2·0	4·9	7·1	8·6	10·3	11·1	11·7							
K	2·2	4·7	6·8	8·4	10·4	10·9	11·6							
NH_4	1·9	4·5	6·6	8·4	10·6	11·3	11·7							

inhibitory in all concentrations from 0·5 to 0·06 *M* and definitely so even at a concentration of 0·008 *M*.

In Fig. 1 a series of experiments with sodium fluoride in varying concentrations shows how the inhibitory action is a function of concentration. Ammonium fluoride is more inhibitory than either sodium or potassium fluoride up to a concentration of 0·03 *M*. Below this strength all three salts have an equal effect.

Chlorides (Table II). In concentrations of 0·5–0·008 *M* the chlorides of lithium, sodium, potassium and ammonium have no effect on the rate of lipolytic action.

Bromides (Table III). With a concentration of 0·5 *M* there is a slight depression of rate, but from 0·25 to 0·008 *M* no effect can be demonstrated.

Iodides (Table IV). As with chlorides, there appears to be no acceleration or retardation of lipolytic action when iodides of lithium, sodium, potassium or ammonium are added in concentrations varying from 0·5 to 0·008 *M*.

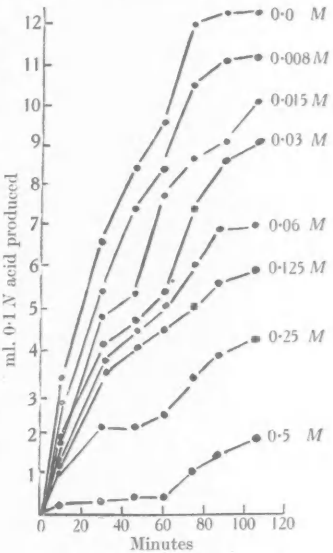

Fig. 1. Sodium fluoride 0–0·5 *M*.

Discussion.

From these results it appears that the halogen salts of lithium, sodium, potassium and ammonium are without effect on the rate of lipase action in concentrations

of 0·5–0·008 *M*, except in the case of the fluorides. Fluorides exert a strongly depressant action which varies directly with concentration of salt. Ammonium fluoride at 0·25–0·06 *M* is more inhibitory than either the sodium or potassium salt, but at 0·5 *M* and from 0·3 to 0·008 *M* all three fluorides show an equal effect.

During the course of these experiments an unexpected effect of halogen salts on the clotting of milk by very weak trypsin was observed. Though the proteolytic enzyme present with the lipase was too weak to show any digestive action on fish-protein, caseinogen or hard-boiled egg-white in five hours, yet in all the concentrations of ammonium fluoride, a junket was formed 15 minutes after the addition of 20 ml. of pancreas substance to 200 ml. milk.

Similar thick soft clots were formed with all four salts of chlorine, bromine and iodine and with potassium and sodium fluorides, except that at the lowest concentration (0·008 *M*) a fine flocculation rather than complete coagulation took place. From these results it seems that the coagulating effect of added calcium salts when rennin, pepsin or trypsin is added to milk may be due to the effect of the acid radical as well as to the metallic ion.

Further experiments on this coagulating action of the halogen salts are in progress.

Summary.

1. The lipolytic activity of pancreas substance is strongly inhibited by sodium, potassium and ammonium fluorides.

2. This inhibition varies directly with the concentration of fluoride.

3. Sodium, potassium and ammonium chlorides and iodides show no effect on the rate of lipase action in concentrations of 0·5–0·008 *M*.

4. Sodium, potassium and ammonium bromides depress lipolytic activity to a slight extent at a concentration of 0·5 *M* but are without effect at 0·25–0·008 *M*.

5. None of the halogen salts, in the concentrations used (0·5 – 0·008 *M*), accelerated lipolytic action.

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LX. A NOTE ON THE INFLUENCE OF p_H ON THE ACTION OF HAEMOLYTIC COMPLEMENT.

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(*Received February 15th, 1934.*)

This problem, which is fundamental to any work on haemolytic complement, does not appear to have received the attention which it deserves. Fuchs [1930] showed that bubbling carbon dioxide through plasma for a short time (6 minutes) increased its complement activity, while bubbling for a longer time (30 minutes) decreased it. He found that the action was increased by carbon dioxide, as long as the reaction was alkaline or neutral to litmus and that it was reduced immediately it became acid to that indicator. It is now thought that CO_2 may have a specific effect on complement, *i.e.* an effect other than through p_H. Andreesco [1930] found that anticomplementary sera had a higher alkaline reserve than normal. This may conceivably be the p_H effect.

Michaelis and Skwirsky [1910] used a series of mixtures of $N/7$ primary and secondary phosphates of sodium and found that specific haemolysis had its optimum at a slightly alkaline reaction corresponding with that of blood. An increase in alkalinity retards haemolysis but not to the extent that the increase in acidity does. Eagle and Brewer [1929] varied the reaction of the medium by the addtion of $N/7$ NaOH and $N/7$ HCl. They found the optimum reaction for haemolysis to be between p_H 6·5 and 8. At p_H 5·3 and in the alkaline range there is an irreversible inactivation, complete at 4·8 and 8·8 respectively.

Unpublished observations indicate that relatively small variations in osmotic pressure affect the speed of complement action. It was therefore decided to repeat these observations with a stricter control of the osmotic pressure of the solution than that of previous workers. A method described by the author was used [Osborn, 1933]. This gives greater accuracy than the serial tube method, used by other observers in this field. It should be noted that this method measures the speed of complement action, whereas the former measures the end results of varying volumes of serum.

In earlier observations an isotonic phosphate buffer was used following Sørensen's method [1909]: this was discarded in favour of a mixture giving a wider range of p_H, *viz.* the "universal buffer mixture" of Prideaux and Ward [1924], as supplied by the B.D.H. Ltd. This mixture—consisting of phosphoric, boric and phenylacetic acids—was made up in twice the concentration recommended by the formula, adjusted to the required p_H by the addition of 0·4N NaOH (this too, being twice the formula strength) and adjusted to approximately the osmotic pressure of corpuscles by the addition of water. Determinations of p_H were made by means of a double hydrogen electrode and of osmotic pressure by measurement of the depression of freezing-point, all solutions being adjusted to within 0·02 % of 0·9 % NaCl.

In determining the speed of complement action $(3·5-x)$ volume of buffer, x volumes of diluted serum and 0·5 volume of a 3 % suspension of sheep's

corpuscles sensitised with 5 units of amboceptor were mixed and placed in a bath at 37°, and the time for the complete haemolysis of the corpuscles was measured. Several different samples of guinea-pig's serum were used in varying concentrations. The guinea-pig's serum and the sheep's corpuscles were supplied by the S.A. Institute for Medical Research; the details of the preparation of the latter have been described previously [Osborn, 1933].

Examples of the results obtained, giving the extreme cases, are recorded graphically in Fig. 1. It will be seen that the optimum p_H is in the region of 7·5

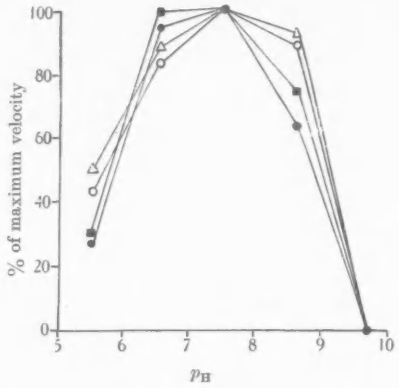

Fig. 1. Showing variation of speed of complement action with p_H.

and that the actual position of the optimum point and the exact shape of the curve vary in different sera, but agree in showing some degree of slowing between p_H 6 and 7, and a marked slowing in the region of p_H 5·5 on the acid side, and a slowing between p_H 8 and 9 and complete inhibition at p_H 9·7 on the alkaline side. These results are in accordance with those of Michaelis and Skwirsky [1910] and Eagle and Brewer [1929]. It is unlikely that more consistent results will be obtained, until it is possible to separate the complement complex from the other constituents of the serum.

Several interesting facts were observed in the course of this work. The buffers themselves retarded haemolysis, for the optimum time with a buffer was at least twice that with normal saline. This retarding influence is common to a wide variety of inorganic and organic ions. (Unpublished observations.)

An attempt to investigate the alkaline range with borate buffers was frustrated by the fact that this ion may be haemolytic to both sensitised and unsensitised corpuscles. The effect appears to be roughly proportional to the hydrogen ion concentration and disappears at about p_H 8. The presence of sodium chloride and other ions protects the corpuscle from this haemolysis.

The observation of Coulter [1921], that heat-inactivation is most rapid in alkaline medium, was confirmed. The haemolytic activity of serum standing at room temperature for 24 minutes at p_H 8·6 was reduced by 44 %, while it suffered no demonstrable change at p_H 7·5, 6·5 or 5·5. That this was a true heat-inactivation was shown by the fact that a control at p_H 8·6 standing in ice-water showed no change.

The author wishes to express his indebtedness to Dr Buchanan of the S.A. Institute for Medical Research for the supply of guinea-pig's serum and sensitised sheep's corpuscles.

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LXI. THE ACTION OF THE CINCHONA AND CERTAIN OTHER ALKALOIDS IN BIRD MALARIA. II.

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(*Received February 16th, 1934.*)

In the previous paper [Goodson *et al.*, 1930], it was shown that Roehl's method of investigating the relative therapeutic efficiencies of antimalarial drugs, by trial in bird malaria, might prove sufficiently delicate to show quantitative differences in action between the eight principal alkaloids of cinchona bark, *viz. l*-quinine, *l*-cinchonidine, *d*-quinidine, *d*-cinchonine (which may conveniently be called the primary cinchona alkaloids) and the reduction products yielded by each of these four bases. In these reduction products the vinyl side-chain of each parent alkaloid is reduced to an ethyl group: they are therefore dihydro-derivatives and are so named throughout this paper. Commercially they are known as hydroquinine, hydrocinchonine, *etc.*

The determination of the relative antimalarial values of the several cinchona alkaloids has now assumed greater practical importance owing to the introduction, by the Health Organisation of the League of Nations, of "totaquina," a product designed as an economical substitute for quinine in the mass treatment of indigent, malarial populations. "Totaquina" is defined [Malaria Commission, League of Nations, 1931], as a mixture of cinchona alkaloids containing a minimum of 15 % of quinine and 70 % of crystallisable cinchona alkaloids, with a maximum of 20 % of amorphous cinchona alkaloids, 5 % of moisture and 5 % of ash.

This innovation, based on the view that there is not a great deal to choose between the crystalline cinchona alkaloids as antimalarial drugs, does not meet with universal acceptance among malariologists, but the opinion of the majority is probably not unfairly represented by the following quotation:

"There is no conclusive evidence to show that any one of the crystallisable alkaloids is so markedly superior to any other as to justify its exclusive use in ordinary cases of malaria in preference to any other, taking into account the shortage of these alkaloids and their cost" [Sinton, 1930].

The misgivings aroused by proposals to substitute for quinine any one of the secondary cinchona alkaloids, or of mixtures such as "totaquina," may be indicated by the following quotation:

"En *premier* lieu il était nécessaire de démontrer que la malaria *peut* être guérie au moyen des alcaloïdes mineurs. Nous n'avons pas besoin d'insister sur ce point qui est, pensons nous, universellement admis.

"En *second* lieu il était nécessaire de donner des préceptes nets au sujet de la façon dont ces alcaloïdes doivent être appliqués pour donner le degré de sécurité auquel on était habitué par un emploi pendant presqu'un siècle de la quinine. Il fallait établir (*a*) à quelles exigences de pureté les nouvelles préparations à

introduire doivent satisfaire et quelle doit être leur composition et (*b*) quelle est la dose à laquelle il faut les administrer pour le succès soit assuré" [Van der Sleen and Drossart Lulofs, 1931].

The definition of "totaquina" allows considerable latitude in the relative proportions of the various cinchona alkaloids present, and in consequence there is much variation in this respect in commercial "totaquina," as the following analyses show:

Table I. *Composition of "totaquina."*

	Type I % *	Type II % †	Type III % *
Quinine	32·5	15·3	17·0
Cinchonidine	34·2	5·7	24·0
Quinidine	—	5·2	10·0
Cinchonine	20·1	55·4	23·0
Total crystallisable alkaloids	86·8	81·6	74·0
Amorphous alkaloids	9·9	13·4	17·0
Moisture	2·6	1·9	5·0
Ash	0·2	2·1	4·0

* Manufacturer's analysis. † Analysis by Mr J. A. Goodson.

It is obvious that if there is a marked difference in the action of, say quinine and cinchonine, the therapeutic values of "totaquina" of types I and II will be different and some evidence for this view has already been brought forward as a result of trials of these two types in bird malaria [Giemsa, 1932] and in human malaria [Malaria Commission, League of Nations, 1933].

It appeared to the authors that definite quantitative evidence on this matter could best be obtained by comparative trials of pure specimens of the individual alkaloids in bird malaria.

The four primary cinchona alkaloids have been purified by processes described later, and a method of controlling these processes, so far as the elimination of the dihydro-bases is concerned has been devised. From the primary bases the pure dihydro-bases have been prepared by catalytic hydrogenation. As there was no certainty that any of these eight alkaloids had been obtained pure previously the principal constants of each have been determined and are recorded and compared with the best of the known previous determinations in Tables II, III and IV.

The biological trials gave results which are summarised in Table A. The figures represent the dose of quinine necessary to produce the same amount of protection as unit dose of the other alkaloids. The figures are only approximate, but the authors think that (*a*) the distinction between quinine and dihydroquinine is certain, (*b*) that dihydroquinidine, cinchonidine and quinidine are definitely less active than quinine and (*c*) that dihydrocinchonine and dihydrocinchonidine form a group of much lower activity than any of the others. The samples of quinidine and cinchonine used were not the purest available; the quinidine contained 1·8 % of dihydroquinidine and cinchonine 1·4 % of dihydrocinchonine. Trials with pure alkaloids were spoilt by the outbreak of a *B. gaertner* epidemic in the stock of canaries, but it will be seen that the removal of these amounts of the dihydro-alkaloids would not affect the order of activity given in the table.

It is not claimed that the order in which the drugs are arranged in this table necessarily represents the order of antimalarial activity in man. But it is logical to assume that, if the alkaloids differ so much in their activity on the parasite experimented with, they will not be of equal therapeutic value in man.

The final decision as to which is the most active must rest on clinical evidence, which is notoriously difficult to assess. It is however, with the present evidence, unjustifiable to assume that all the crystallisable alkaloids are of equal value.

The same order can be deduced from the results of previous workers [Goodson *et al.*, 1930; Giemsa *et al.*, 1926; Giemsa and Oesterlin, 1933] except as regards quinidine. Goodson *et al.* [1930] obtained results, which suggest that quinidine has a higher activity than that now assigned to it. The number of birds on which quinidine is given its place, is small, owing to the fact that some died during the experiment. It is probable that 5 mg. daily is too toxic a dose for quinidine, though the large majority of canaries survive these doses of the other alkaloids. In this respect our canaries are less sensitive than those used by Giemsa. The apparent difference cannot be explained by any difference in purity of the alkaloids used. The point is however being further investigated together with the proper place of cinchonine.

The effect of hydrogenation is astonishingly different for different alkaloids. For quinine and possibly quinidine, hydrogenation increases the activity; for cinchonine the effect is probably to diminish the activity. No possible explanation can be offered and yet another instance of lack of correspondence between biological activity and chemical structure must be left on record.

Table A.

Dihydroquinine	1–2	Quinidine	About 0·5*
Quinine	1	Cinchonine	Less than 0·2
Dihydroquinidine	0·5–1	Dihydrocinchonine	,,
Cinchonidine	About 0·5	Dihydrocinchonidine	,,

* Further experiments with the pure alkaloids suggest that this is an overestimate rather than an underestimate of the relative activity of quinidine.

Modified cinchona alkaloids. In the previous paper it was shown that although quitenine is itself inactive it recovers antimalarial activity on esterification; this activity is slight for the methyl and ethyl esters and only becomes well-marked in the butyl and amyl esters. Observations with two of these esters have been repeated with the results recorded in Table B, which are given in a form readily comparable with those of Goodson *et al.*

These results are interesting in two ways; (1) they are both positive, whereas in the former more extensive series negative results were occasionally obtained with each substance; (2) the improvement in activity from ethyl to amyl is not so well-marked as in the previous experiments. These alkylquitenines are somewhat readily hydrolysed, and it is suggested that the apparent variation in antimalarial activity is caused by varying capacity for hydrolysing the drugs shown by different canaries. Less variable results would probably be obtained by intravenous injection, a method not well suited for use with canaries.

Certain of the cinchona alkaloid "chlorides" being available for other purposes, the opportunity was taken to try them in bird malaria. Quinine chloride, dihydroquinine chloride and cinchonidine chloride all proved inactive in six daily doses of 5 mg. as was to be expected from the recorded inactivity of quinine chloride [Giemsa *et al.*, 1926]. It is however of interest to note that whilst in the authors' experiments no difficulty was experienced in giving doses of 5 mg. of these chlorides, Giemsa was only able to give 1·5 mg., a further example of variability in canaries. The disappearance of activity as a result of replacing the —CHOH— group by —CHCl— is a striking example of the important part played by this group in this set of alkaloids, activity disappearing

on its conversion into —CO— (quininone), —CH_2— (desoxyquinine), :CH— (quinene) or —CH.OAc— (acetyldihydroquinine) [Giemsa and Oesterlin, 1933] and no change so far effected has left the activity unimpaired.

Table B.

Substance	Daily doses: No.	Daily doses: Quantity mg.	Retardation days
Ethylquitenine hydrochloride	6	5	5
iso-Amylquitenine dihydrochloride	6	5	6

The authors desire to place on record their indebtedness to Prof. van der Sleen of the Kina Bureau, Amsterdam, for information on a number of technical points connected with the analysis of cinchona alkaloids. They also owe thanks to Mr W. Solomon, B.Sc., for the preparation of the three cinchona alkaloid chlorides mentioned, to Messrs A. C. Camfield, E. M. Gibbs, and N. J. G. McLaren for much patient work on the fractionation and biological tests of the alkaloids, and to Messrs A. Bennett and H. C. Clarke for the micro-analyses.

Chemical section.

Crystallisable cinchona alkaloids. The impurities in commercial samples of the primary cinchona alkaloids are of two kinds. Each alkaloid contains its own reduction product, which in the case of quinidine is now shown to be present to the extent of 20 % and may be as high as 30 % [Dawson and Garbade, 1930], while cinchonine usually contains about 14 % of dihydrocinchonine. These remarks apply to well-crystallised specimens of these alkaloids, usually accepted by chemists and biologists as pure owing to their constancy of melting-point and optical rotation. The amounts of dihydro-bases in quinine and cinchonidine are much less but are not negligible. The other type of impurity is due to the difficulty of completely separating the components of each of the two similarly optically active pairs from each other, *i.e.* cinchonidine and quinine usually contaminate each other, as do quinidine and cinchonine, but components of the laevorotatory pair are not usually found in those of the dextrorotatory pair and the converse is also true.

The purification of any one of the four primary alkaloids thus involves two steps: (1) the removal of the second component of the similarly optically active pair, *e.g.* cinchonidine from quinine and *vice versa*, and (2) the elimination from each alkaloid of the dihydro-base, which naturally accompanies it.

Much work has been expended in attempts to prepare each of the primary cinchona alkaloids in a pure state, but only two of the numerous papers on this subject need be referred to here [Tutin, 1909; Emde, 1932]. Tutin prepared pure quinine by repeated crystallisation of the acid sulphate, but was unable to prove that it was free from dihydroquinine. This proof is now supplied. Emde set out to prepare all eight alkaloids in a pure state, specially recommending for this purpose crystallisation of the dihydrobromides, but he did not prove the absence of the dihydro-bases from his final specimens of the four primary alkaloids.

In the course of the present investigation, pure specimens of each of the primary alkaloids have been prepared, the chief criteria of purity adopted (apart from well-known specific tests, such as fluorescence, methoxyl determinations and the thalleioquin reaction) being (*a*) agreement of the "found" and "calculated" values for hydrogen absorbed, by the conversion of the vinyl group into

an ethyl group, under standard, quantitative conditions, as proof of the absence of dihydro-bases and (*b*) constancy of optical rotation of the base and its salts, when expressed on a uniform basis and taken under standard conditions. The chief effects of this purification are to remove quinine from "cinchonidine" and quinidine from "cinchonine," and to eliminate large quantities of dihydro-bases from what have hitherto been commonly regarded as pure cinchonine and pure quinidine. From the pure primary alkaloids the corresponding dihydro-bases have been made by catalytic hydrogenation.

The chief constants of the eight pure alkaloids so prepared are recorded in Tables III and IV.

Though numerous determinations of the specific rotations of the cinchona alkaloids are recorded, they are for such varied concentrations and solvents, that it is not practicable to use them for comparative purposes. The authors have however repeated their determinations for the dihydrobromides of all eight alkaloids, under the conditions used by Emde in order to establish a more exact comparison with the results of that author. These comparative results are given in Table II.

Table II. *Specific rotations of dihydrobromides of cinchona alkaloids.*

Base	Salt used	c (anhydrous salt)	Solvent	$[\alpha]_D$ recorded by Emde	$[\alpha]_D$ recorded by Present authors
Quinine	B.2HBr, $3H_2O$	3·5194	Water	−181·04°*	−184·4°
Dihydroquinine	B.2HBr, $3H_2O$	3·902	Water	−152·7	−153·2
Cinchonidine	B.2HBr, $2H_2O$	4·2575	Water	−112·5	−111·4
Dihydrocinchonidine	B.2HBr, $2H_2O$	2·0828	Water	− 91·9	− 90·6
Quinidine	B.2HBr, $3H_2O$	3·6163	Water	+209·5 to +210·7	+219·2
Dihydroquinidine	B.2HBr, $3H_2O$	3·6628†	Water + HBr	+196	+197·5
Cinchonine	B.2HBr	4·2355	Water	+163·2	+166·6
Dihydrocinchonine	B.2HBr	5·0233	Water	+142	+143·1

* Emde gives $[\alpha]_D^{25°}$ −183·6° for *c* 3·9363.

† Average of 7 determinations: *c* = 3·6491 to 3·6836 and $[\alpha]_D^{22 \text{ to } 27°}$ = 193·2 to 197·6°.

It will be seen that the two sets of figures are in good agreement so far as cinchonidine and the four dihydro-bases are concerned, the differences ranging from 0·5 to 1·5, which are not greater than might be expected from two workers, making observations on the same substance. With quinine the difference amounts to 3·36°. Emde purified his quinine, first as the acid sulphate and finally as the dihydrobromide and there can be no doubt as to its purity. It is suggested that his lower specific rotation may be due to incomplete drying. Thus, he describes quinine dihydrobromide as a dihydrate, but the present authors found that the loss on drying at 120° *in vacuo* in seven samples varied from 10·12 to 10·86 % (average 10·37 %). The loss calculated for a trihydrate is 10·0 % and for a dihydrate 6·9 %.

In the case of cinchonine the difference is 3·4°. In this instance the salt is anhydrous and the higher figure now recorded is doubtless due to the complete elimination of dihydrocinchonine. Assuming this, the lower figure recorded by Emde corresponds to a cinchonine containing 14·3 % of dihydrocinchonine, which is close to the figure 15·1 % now found (p. 437) for commercial cinchonine after recrystallisation from alcohol until free from quinidine.

The figures for quinidine show the largest difference 8·5 to 9·7°. Emde found quinidine dihydrobromide to be a dihydrate whereas the present authors found

it to be a trihydrate. It is possible that in this case also Emde's lower figures are in part due to incomplete drying. Assuming that Emde really used a monohydrate instead of the anhydrous salt for his determination, his figure would be raised to $+217{\cdot}9°$, which on the basis of the present authors' results would correspond to quinidine containing 6 % of dihydroquinidine. This calculation assumes that the change in rotation due to dilution is the same and remains constant for quinidine and dihydroquinidine dihydrobromides. This is approximately true so far as the dilutions from $M/10$ to $M/40$ given in Table III are concerned, the two figures being 3·7 and 3·9°. The same assumption is made in the calculation for cinchonine given above and in this case the difference due to dilution of the dihydrobromides from $M/10$ to $M/40$ is 2·6° for both cinchonine and dihydrocinchonine.

Two further comparisons are possible with the results recorded in Table III. The Dutch Pharmacopoeia permits a range of $-17{\cdot}7°$ to $-18°$ for the observed rotation of $M/10$ solutions of quinine and its salts taken under specified conditions as to solvent and temperature. Under these conditions $M/10$ solutions of the quinine salts described in Table III gave the following results: monohydrochloride $-18{\cdot}06°$; neutral sulphate $-18{\cdot}095°$ and acid sulphate $-18{\cdot}085°$.

Realising the lack of any method of eliminating the large quantity of dihydroquinidine present in ordinary quinidine, a successful attempt was made recently [Butler and Cretcher, 1933] to prepare pure quinidine by re-arrangement of quinine [Rabe *et al.*, 1932]. The yield was apparently small. The product had $[\alpha]_D+323{\cdot}8°$ ($c=2$ in 1·8 % HCl). The pure quinidine used in the present investigation had $[\alpha]_D+323{\cdot}1°$ observed under the same conditions.

The specific rotations, calculated as base in Tables III and IV, bring out the interesting point that all eight alkaloids in the form of dihydrobromides show a lower specific rotation in $M/10$ than in $M/40$ solution in water. It has not been possible to extend this comparison to other acid salts of all eight bases owing to the difficulty of getting satisfactory preparations of the complete series; the acid sulphates, for example, are too soluble to yield good preparations in some instances unless large quantities of the pure bases are available, and in other cases they cannot be dried completely without decomposition. It is however of interest to note that whilst quinine acid sulphate, like the dihydrobromide, shows a higher rotation at $M/40$ than at $M/10$, cinchonidine acid sulphate shows the same specific rotation at both concentrations. Determinations of p_H of these solutions, for which the authors are indebted to Mr C. G. Pope, throw no light on these differences. Anomalies of this kind in connection with the optical rotation of cinchona alkaloids have long been recorded [Oudemans, 1876] and have frequently been investigated, but so far no satisfactory and final explanation of their occurrence has been given.

Preparation of the drugs.

Separation of the primary alkaloids. It is easy to detect mere traces of quinine in cinchonidine, or of quinidine in cinchonine by the fluorescence of solutions in dilute sulphuric acid; and in purifying cinchonidine and cinchonine, each base was crystallised until a 1 % solution in $N/10$ sulphuric acid examined in bright, diffused daylight in a 2 dm. polarimeter-tube, showed no greater intensity of fluorescence than a 1 in 1,000,000 solution of quinine or quinidine respectively, examined in like manner. Under these conditions the fluorescence of quinine is just visible at 1 in 2,000,000.

The inverse operation, *viz.* the detection of cinchonidine in quinine has been much investigated, and in practice there are two methods in use, *viz.* the

ammonia test, which probably does not detect less than 2 % of cinchonidine, and the polarimetric test, which is of no value for the present purpose, since quinine is contaminated not only with cinchonidine, but also with the optically active bases, dihydroquinine and dihydrocinchonidine. Cinchonidine is less soluble in benzene than quinine, whilst its acid salts are more soluble in water than those of quinine: there is no reason to doubt that by rejection of appropriate fractions in fractional crystallisation from such solvents the small amount of cinchonidine in commercial quinine is eliminated.

The detection of cinchonine in quinidine is also difficult; there is a considerable difference between the optical rotations of the two alkaloids, but there has been until now no method of distinguishing between cinchonine and dihydroquinidine as the cause of low rotation in commercial quinidine. Cinchonine is less soluble in alcohol than quinidine and, it is shown later (p. 434) that it can be eliminated from crude quinidine by recrystallisation from boiling alcohol, the first fractions being rejected and any traces that escape in this process being left in the mother-liquors in the final fractionation, cinchonine dihydrochloride being much more soluble in water than quinidine dihydrochloride.

Elimination of dihydro-bases. The second group of impurities consists of the naturally-occurring dihydro-alkaloids, each primary alkaloid being accompanied by its own reduction product. The vinyl group in each of the parent bases is readily oxidised to a carboxyl group by permanganate, while the reduced bases are relatively stable to this reagent, and this method has hitherto been used both for the isolation of these dihydro-bases and for their estimation. Since the dihydro-bases are only relatively stable to permanganate, such a method can merely give approximate results and for the detection and estimation of the dihydro-bases, the authors have used a new method, *viz.* catalytic hydrogenation in an apparatus designed for quantitative work [Paget and Solomon, 1932]. The errors in such a determination are estimated not to exceed ± 1 %, and a primary cinchona alkaloid has been regarded as free from its dihydro-base when it absorbed 99·5 to 100·5 % of the quantity of hydrogen required to convert the vinyl group into an ethyl group. Colloidal palladium on barium sulphate or Adams's platinic oxide has been used as a catalyst, the former being preferred as it is less liable to form colloidal solutions from which the reduction product is difficult to recover. For the estimations, 0·4 to 0·5 g. of the base or one of its salts dissolved in 10 ml. of $N/10$ sulphuric acid, containing 0·05 g. of palladised barium sulphate, is exposed to hydrogen with constant, vigorous mechanical agitation. Absorption is rapid during the first half hour and finishes in about 2 hours after which it is taken as complete if, during a further 2 hours, the change in volume of gas is not greater than 0·1 ml. If, after this point is reached, action is continued, there is a slow absorption of 0·2 to 0·5 ml. *per diem* due no doubt to the very slow formation of more highly hydrogenated bases, but there is no practical difficulty in determining the point at which rapid formation of the dihydro-base is complete and the second very slow absorption begins. The results for hydrogen absorption given below are corrected for hydrogen absorbed by the catalyst, which must be determined by blank experiments on each new batch of catalyst made.

The bulk samples of dihydro-bases used were made by catalytic hydrogenation in like manner of the purified primary alkaloids and were crystallised as bases or salts to constant optical rotation. As so much attention has been given to the permanganate test for the dihydro-bases, it was thought desirable to use this in a more or less quantitative form. For that purpose, 5 ml. of a 2 % solution of the dihydro-base in water containing 0·3 ml. of N sulphuric acid was

treated with 0·5 ml. of $N/50$ potassium permanganate. The colour disappears in not less than 10 minutes with all the dihydro-bases except dihydrocinchonine, which discharges it in 30 seconds. The specific rotations of the pure bases and their salts are recorded in Tables III and IV. It is well known that in this series the specific rotation varies with the nature of the solvent and the concentration of the solution. As the authors had to make extensive use of polarimetric observations to follow the course of crystallisation in the final purification of the alkaloids, they found it convenient to use $M/40$ solutions of any salt selected for this purpose, since the series of readings were then directly comparable without calculation. The figures recorded for $[\alpha]_D$ in Tables III and IV are in all cases for $M/40$ or $M/10$ solutions of the dry salt or dry base in water or $0{\cdot}1N$ acid, usually $0{\cdot}1N$ sulphuric acid.

Additive compounds of cinchona alkaloids with metallic salts. The elimination of the dihydro-bases from quinine and cinchonidine is easy, and from cinchonine and quinidine slow and laborious. In the hope of improving it numerous salts and derivatives were tried. Quinidine sulphate argentonitrate, $B_2.H_2SO_4, AgNO_3$, proved useless, because it could not be recrystallised without decomposition. The zincichlorides of the type $B.(HCl)_2, ZnCl_2$, can be recrystallised, are easily purified, and the alkaloids are readily recoverable from them, but they are formed with equal facility and are of similar solubility with both the parent and the reduced bases and are therefore of no value as a means of separation. When this work was almost completed, a paper was published [Cohen, 1933] describing the specific precipitation by cupric chloride of cinchona alkaloids containing the vinyl group, and making the statement that "no complex salts are precipitated when the corresponding dihydro-bases are treated with cupric chloride under exactly the same conditions, even after several weeks at $-4°$." As the cuprichlorides are represented by the formula $B.(HCl)_2, CuCl_2$, which is strictly analogous with that found for the zincichlorides, it seemed curious that this specificity should be exhibited by cupric chloride and not by zinc chloride. The behaviour of cupric chloride with commercial cinchonine and commercial quinidine was investigated carefully and it was found that the cuprichlorides did, in fact, effect a partial separation of the dihydro-bases. The resulting fractionation showed clearly that the dihydro-bases must form insoluble cuprichlorides and, on trying the experiment, no difficulty was experienced in preparing cuprichlorides of all four dihydro-bases, except in the case of dihydrocinchonidine, where the additive compound is readily soluble in conc. HCl at atmospheric temperatures and when prepared in small quantities is best manipulated in a cold room.

In the following account, the melting-points quoted are corrected; the combustion results are for material dried at 120° *in vacuo* unless otherwise stated and were obtained by micro-analysis.

Quinine. The material used consisted of base prepared from commercial quinine sulphate. It had $[\alpha]_D^{15°} - 280{\cdot}7°$ ($c = M/40$ in $N/10$ H_2SO_4) and hydrogen absorption 94·7 %. It is known that cinchonidine can be eliminated from quinine by repeated crystallisation of the acid sulphate [Tutin, 1909]. The authors have found that quinine purified in this way is also free from dihydroquinine, as indicated by its absorptive capacity for hydrogen. Purification can also be effected by crystallisation of the dihydrobromide [Emde, 1932]. The quinine acid sulphate was prepared by repeated crystallisation (five or six times is usually necessary) from hot water (1 in 7 to 8) until the rotation became constant. It was then recrystallised in three fractions, for which $[\alpha]_D^{15°}$ showed the narrow range $-216{\cdot}1°$ to $-217{\cdot}0°$ ($c = M/40$ in water); the hydrogen absorption, for which the middle fraction was used, was 100·1 %. The dihydrobromide was prepared by recrystallisation from hot (43°) water (1 in 3 to 4) with the same precautions as the acid sulphate: the range of specific rotation for the three final fractions was $-189°$ to $-189{\cdot}6°$, the hydrogen

absorptions of the middle fractions were 100·8, 101·5 and 101·6 % (hydrogen absorptions by quinine dihydrobromide were generally high). The neutral hydrochloride and sulphate, made by neutralising pure quinine base with the appropriate acid, were recrystallised from hot water containing a trace of the appropriate acid to avoid any risk of inclusion of free base, which accounts for the high rotation shown by some specimens of neutral quinine salts.

Constants for the pure base and salts are given in Table III.

Dihydroquinine. The base, prepared by hydrogenation of pure quinine was crystallised from hot benzene, or as the neutral sulphate or the dihydrobromide from hot water. Constants for these three preparations are given in Table III.

Dihydroquinine cuprichloride. Dihydroquinine (5 g.) was dissolved in conc. HCl (8 ml.) and cupric chloride (5 g.) in conc. HCl (8 ml.) added: the mixture was kept at 7° for 24 hours, but nothing separated. The side of the beaker just under the surface of the liquid was then rubbed vigorously with a glass rod for 5 minutes. A yellow streak appeared and in 20 minutes the liquid had set to a semi-solid, crystalline magma. The crystals were filtered out on Whatman filter-paper No. 54, washed sparingly with conc. HCl and dried in a vacuous desiccator over soda lime; wt. 7·0 g. The product was recrystallised from 14 ml. hot conc. HCl; wt. 3·9 g. It crystallised on the sides of the container in silky, cushion-like masses of golden-brown needles, M.P. 204° (decomp.). (Found: C, 44·61; H, 5·22; N, 5·26; Cl, 26·60; Cu, 11·68 %. $C_{20}H_{26}O_2N_2.2HCl, CuCl_2$ requires: C, 44·98; H, 5·28; N, 5·25; Cl, 26·57; Cu, 11·9 %.)

Dihydroquinine zincichloride. $C_{20}H_{26}O_2N_2.2HCl, ZnCl_2$. The base (5 g.) dissolved in conc. HCl (8 ml.) was mixed with zinc chloride (12·5 ml. of 40 % solution in conc. HCl). Crystallisation began almost immediately, and the product was isolated in the same way as the cuprichloride. Yield 6·9 g. It crystallises from hot water (1 in 5) but shows a tendency to separate as an oil, which can be corrected by the addition of a little alcohol; also from hot 50 % alcohol or from hot conc. HCl in masses of colourless, flattened prisms, M.P. 257°; froths 262°. (Found: C, 45·12; H, 5·43; N, 5·28; Cl, 26·35; Zn, 12·20 %. Calc.: C, 44·82; H, 5·27; N, 5·23; Cl, 26·48; Zn, 12·22 %.)

Quinidine. The quinidine base used had $[\alpha]_D^{15°}$ +324·3° ($c = M/40$ in $N/10$ H_2SO_4) and hydrogen absorption 77·9 % indicating the presence of 22·1 % of reduced bases, chiefly dihydroquinidine. After recrystallisation from boiling alcohol, the first fraction (about 10 %) being rejected, it had $[\alpha]_D^{15°}$ +325·5° and hydrogen absorption 76·4 %; these slight changes are accounted for by removal of cinchonine. The hydrogen absorption could not be raised by crystallisation of either the base from alcohol, or the dihydrobromide from water, but the dihydrochloride (1 in 1 in hot water) proved suitable for this purpose, the less soluble fractions being richest in quinidine, as indicated by increasing rotation and hydrogen absorption. Three such crystallisations give a yield of about 33 % by weight of the starting material, and raise the hydrogen absorption to 90 %. This procedure answers well until a product having hydrogen absorption 98·0 to 98·5 % is reached. At this stage crystallisation from weaker solutions (1 in 3) ensures more rapid purification. At the first point at which approximate purity is reached, the yields are about 2·75 % of material with hydrogen absorption 100·6 % and about 7·5 % showing hydrogen absorption from 98 to 99 %.

In the course of attempts to find a derivative of quinidine, which would expedite the tedious fractional crystallisation necessary especially in the last stages of purification by means of the dihydrochloride, addition compounds of the alkaloid with silver nitrate and with zinc chloride were examined.

Quinidine argentonitrates. Two silver nitrate addition products of quinidine are known, one represented by the formula $(C_{20}H_{24}O_2N_2)_2, AgNO_3$ [Stenhouse, 1864], which cannot be recrystallised without decomposition, and a second, obtained as a crystalline precipitate when silver nitrate is added to dilute solutions of quinidine sulphate. Though used as a test for quinidine, the origin of this latter reaction has not been traced, and the nature of the precipitate appears to be unknown. It was prepared by adding silver nitrate solution (5 ml. of 50 %) to a boiling, aqueous, faintly acid solution of quinidine sulphate (5 g.). As the solution cooled, colourless needles of quinidine sulphate argentonitrate separated. Yield 6·2 g. The substance is sparingly soluble in boiling water, but can be crystallised with some decomposition from boiling 50 % alcohol in colourless needles containing $2H_2O$. Loss at 120° *in vacuo* 3·9: calc. 3·7 %.) M.P. 213° (decomp.). (Found: C, 51·3; H, 5·98; N, 7·72; MeO, 6·32; Ag, 11·74; S, 3·51 %. $(C_{20}H_{24}O_2N_2)_2.H_2SO_4, AgNO_3$ requires: C, 52·3; H, 5·4; N, 7·6; MeO, 6·7; Ag, 11·7; S, 3·49 %.) The carbon determinations were per-

sistently low in recrystallised specimens, owing to the slight decomposition observed on recrystallisation, but there can be little doubt that the substance is correctly represented by the formula given. The "quinidine" recovered from the silver nitrate compound absorbed 81·1 % of the calculated amount of hydrogen, which is only a slight improvement on the original figure of 78 %.

Quinidine zincichloride. This was made by the usual method (p. 434) using quinidine 1 in 5 and zinc chloride 1 in 3 of conc. HCl. It crystallises from hot water or 50 % alcohol but best from hot conc. HCl (1 in 3) when it forms much-twinned colourless prisms, M.P. 242° (decomp.). In the following analyses the two figures for each constituent are for material crystallised from conc. HCl and water respectively and dried at 120° *in vacuo*. (Found: C, 44·98, 45·0; H, 5·13, 5·10; N, 5·32, 5·49; MeO, 6·02, 6·23; Cl, 25·9, 25·48; Zn, 12·5, 12·4 %. $C_{20}H_{24}O_2N_2.2HCl, ZnCl_2$ requires: C, 44·9; H, 4·87; N, 5·25; MeO, 5·8; Cl, 26·5; Zn, 12·2 %.) This is the formula adopted by Stenhouse for what was undoubtedly the same substance and the foregoing confirms and extends his description.

Quinidine cuprichloride. In view of the claim [Cohen, 1933] that the vinyl-containing cinchona alkaloids form solid cuprichlorides whilst the dihydro-bases do not, this method was tried on quinidine. The "quinidine" was not recovered by Cohen's method of removing copper as the sulphide but by the simpler process of dissolving and thereby decomposing the cuprichloride in cold water and pouring the filtered solution into excess of ammonia solution, when the base is precipitated and is readily filtered and washed free from the ammoniacal copper solution. The following is a typical result of a close imitation on a larger scale of one of Cohen's experiments with the modification just referred to, starting with a "quinidine" fraction having a hydrogen absorption of 51·6 %: Cohen used a mixture, quinidine-dihydroquinidine (1:1).

The "quinidine" (11 g.) was dissolved in conc. HCl (40 ml.) and cupric chloride (11 g.) in 36 ml. hot conc. HCl added. On cooling, the orange-coloured crystalline cuprichloride separated and was collected, dried overnight on a porous tile and finally in a vacuous desiccator over soda lime for 48 hours. Yield 11 g. A filtered aqueous solution of the cuprichloride (3 g. in water 30 ml.) was run into solution of ammonia (10 ml. conc. NH_4OH with water 30 ml.) and the precipitated base collected, thoroughly washed and dried in a vacuous desiccator. Yield 1·97 g. It absorbed 67·5 % of the calculated amount of hydrogen, *i.e.* an improvement of 15·9 %. In other trials with varying amounts of hydrochloric acid as solvent such results as the following were obtained; the figures are the hydrogen absorption values % of (*a*) the "quinidine" used, and (*b*) the recovered "quinidine."

(*a*)	77·9	77·9	86·6	78·6	80·4	88·1	95·8
(*b*)	91·0	88·0	88·7	80·4	88·1	95·8	98·0

The method has obvious possibilities and by combining it with the present authors' method—recrystallisation of the dihydrochloride—it is possible to obtain the latter in a pure state. The remainder of the "quinidine" cuprichloride (8 g.) prepared from the 51·6 % "quinidine" referred to above, was recrystallised from hot conc. HCl (1 g. in 3 ml.) and the whole of the crop (4·6 g.) ground in a mortar with water (9 ml.). Under these conditions the cuprichloride decomposed, and part of the quinidine dihydrochloride formed crystallised out. This was collected and without washing or drying was converted into base as already described. Yield 2·15 g. This absorbed 85·2 % of the calculated amount of hydrogen, an improvement of nearly 34 %. Still better quinidine but in poorer yield can be obtained by the use of more water (3 or 4 ml. instead of 2 ml. for 1 g. of cuprichloride). The base recovered from the dihydrochloride mother-liquors is of poorer quality.

Starting with "quinidine" showing hydrogen absorption 77·9 % and using conc. HCl (5 ml.) for each g. of quinidine or cupric chloride employed, it is possible by one treatment by this modified process, to obtain 1/3 of the original amount as a fraction showing hydrogen absorption about 95·4 % and by repeating the operation to obtain about 1/9 of the original amount having hydrogen absorption 99 to 100 %.

The quinidine used in determining the constants of the base, acid sulphate and dihydrobromide recorded in Table III was made by both processes. No figures are given for quinidine dihydrochloride as this substance is not completely dehydrated at 120° *in vacuo*, and between 120° and 130° the loss is irregular owing to incipient decomposition.

Dihydroquinidine. This base, prepared by catalytic hydrogenation of pure quinidine was crystallised from boiling alcohol, or as the dihydrobromide from water.

Dihydroquinidine cuprichloride was prepared by mixing the base (2 g.) with cupric chloride (2 g.) each having been dissolved separately in conc. HCl (5 ml.). Nothing separated on standing for 24 hours except a few crystals of cupric chloride, but on rubbing the side of the beaker, yellow granules appeared and the whole liquid quickly became semi-solid. Wt. 1·71 g. It was recrystallised from hot conc. HCl (1 in 1·5 ml.) from which it separated in clusters of thin, dark orange, rectangular prisms, M.P. 222° (decomp.). Yield 0·9 g. (Found: C, 44·94; H, 5·37; N, 5·17; Cl, 26·55; Cu, 11·92 %. $C_{20}H_{26}O_2N_2.2HCl, CuCl_2$ requires: C, 44·98; H, 5·28; N, 5·25; Cl, 26·57; Cu, 11·91 %.)

Dihydroquinidine zincichloride was prepared by mixing the base (2 g.) in conc. HCl (5 ml.) with zinc chloride (5 ml. of 40 % solution in conc. HCl). Nothing crystallised out until the inside of the beaker was rubbed vigorously with a glass rod. Wt. 1·7 g. It can be recrystallised from water, 50 % alcohol or hot conc. HCl (1 g. in 2 ml.) and separates from the latter in colourless, spheroidal masses of minute silky needles, M.P. 249–252° (decomp.). (Found: C, 44·6; H, 5·31; N, 5·19; Cl, 26·45; Zn, 12·00 %. $C_{20}H_{26}O_2N_2.2HCl, ZnCl_2$ requires: C, 44·82; H, 5·27; N, 5·23; Cl, 26·48; Zn, 12·22 %.)

Constants for dihydroquinidine and its salts are given in Table III.

Table III. *Constants of pure cinchona alkaloids.*

Methoxy-bases: quinine, dihydroquinine; quinidine, dihydroquinidine.

Substance	α_D	c*	Solvent	$[\alpha]_D^{15°}$ calc. for Salt	$[\alpha]_D^{15°}$ calc. for Base	Additional constants
Quinine (Q):						
Base, M.P. 173·5°	− 4·611°	*M*/40	*N*/10 H_2SO_4	—	−284·5°	H.A.† = 99·93
Acid sulphate, $Q.H_2SO_4, 7H_2O$	−17·875	*M*/10	Water	−211·7°	−275·9	H.A. = 100·1: p_H = 2·86
,, ,,	− 4·555	*M*/40	,,	−216·1	−281·4	p_H = 3·08
,, ,,	− 4·607	*M*/40	*N*/10 H_2SO_4	−218·3	−284·3	—
Neutral sulphate, $Q_2.H_2SO_4, 7H_2O$	− 4·608	*M*/40	*N*/10 H_2SO_4	−247·1	−284·4	—
Dihydrobromide, $Q.2HBr, 3H_2O$	−17·890	*M*/10	Water	−184·1	−276·2	H.A. = 100·8: p_H = 2·61
	− 4·608	*M*/40	,,	−189·6	−284·4	p_H = 2·94
Neutral hydrochloride, $Q.HCl, 2H_2O$	− 4·597	*M*/40	*N*/10 H_2SO_4	−255·1	−283·8	—
	− 4·582	*M*/40	*N*/10 HCl	−254·3	−282·9	—
Dihydroquinine (HQ):						
Base, M.P. 173·5°	− 3·842	*M*/40	*N*/10 H_2SO_4	—	−235·7	—
Dihydrobromide, $HQ.2HBr, 3H_2O$	−14·880	*M*/10	Water	−152·5	−228·3	—
	− 3·845	*M*/40	,,	−157·5	−235·8	—
Neutral sulphate, $(HQ)_2.H_2SO_4, 6H_2O$	− 3·838	*M*/40	*N*/10 H_2SO_4	−204·6	−235·4	—
Quinidine (Qd):						
Base, M.P. 173·5°	+ 5·415	*M*/40	*N*/10 H_2SO_4	—	+334·2	H.A. = 99·8 %
Acid sulphate, $Qd.H_2SO_4, 4H_2O$	+20·915	*M*/10	Water	+247·8	+322·7	—
	+ 5·409	*M*/40	*N*/10 H_2SO_4	+256·4	+333·9	—
Dihydrobromide, $Qd.2HBr, 3H_2O$	+21·315	*M*/10	Water	+219·3	+328·9	—
	+ 5·420	*M*/40	,,	+223·0	+334·5	—
Dihydroquinidine (HQd):						
Base, M.P. 169·5°	+ 4·875	*M*/40	*N*/10 H_2SO_4	—	+299	—
Dihydrobromide, $HQd.2HBr, 3H_2O$	+19·178	*M*/10	Water	+196·5	+294·2	—
	+ 4·890	*M*/40	,,	+200·4	+300	—

* c is expressed as dry substance.
† H.A. = hydrogen absorption: % of calculated value (p. 432).

Cinchonidine. Commercial cinchonidine is stated to contain quinine 10 % and dihydrocinchonidine 8 % [Dawson and Garbade, 1930]. The base, used as a starting material, showed strong fluorescence on solution in dilute sulphuric acid, had $[\alpha]_D^{15°}$ − 183·2° ($c = M/40$ in $N/10$ H_2SO_4) hydrogen absorption 97·9 % and contained methoxyl 0·66 %. These results indicate the presence of quinine (about 6·9 %) and dihydrocinchonidine (about 2 %).

It was found that crystallisation three times from boiling benzene (1 in 30), the first fractions only being collected, yielded about 78 % of a product, which passed the fluorescence test (p. 431) and in which no methoxyl could be detected. This, on fractional crystallisation from alcohol

(1 in 6), yielded first and third fractions having hydrogen absorptions of 98 to 99 % and a middle fraction with hydrogen absorption 101·2 %, the yield of the latter being about 25 % of the original material. In some cases it was necessary to crystallise the quinine-free product twice from alcohol, in order to reach this degree of purity. The constants for the base, acid sulphate and dihydrobromide are recorded in Table IV.

Dihydrocinchonidine. The base prepared by hydrogenation of pure cinchonidine was recrystallised from boiling benzene and finally from alcohol, or as the dihydrobromide from water. The constants are given in Table IV. The *cuprichloride* was prepared by mixing the base (3 g.) in conc. HCl (4 ml.) with cupric chloride (3 g.) in conc. HCl (5 ml.). Even after vigorous rubbing of the cooled liquid with a glass rod no crystals appeared, but after 24 hours at 7°, a crop of minute pale, canary-yellow needles separated, wt. 2·6 g. It was recrystallised in the same way from warm conc. HCl (3 ml.), wt. 1·5 g., and was dried on a porous tile. It sinters at 120–140°, melts at 166° and froths at 183°. (Found on substance dried at 90° *in vacuo*: C, 44·52; H, 5·29; N, 5·5; Cl, 28·15; Cu, 12·83 %. $C_{19}H_{24}ON_2.2HCl, CuCl_2$ requires: C, 45·27; H, 5·20; N, 5·56; Cl, 28·16; Cu, 12·62 %.)

Cinchonine. The commercial cinchonine used was markedly fluorescent in dilute sulphuric acid, contained methoxyl 0·25 %, corresponding to quinidine 2·6 % and had $[\alpha]_D^{17}$ +264·5° ($c = M/40$ in $N/10$ H_2SO_4) and hydrogen absorption 86·2 % indicating the presence of 13·8 % of dihydrocinchonine. After three crystallisations from boiling alcohol (1 in 30) it passed the fluorescence test (p. 431) and the figure for hydrogen absorption fell to 84·9 owing to removal of quinidine. This partially purified base was converted into dihydrobromide and recrystallised from water (1 in 1). The first crop, which is rich in dihydrocinchonine is rejected and successive crops are obtained by slow concentration of the mother-liquor in a vacuous desiccator over sulphuric acid. When a conveniently large crop has accumulated in this way, it is scraped down into the mother-liquor, dissolved by warming and left to crystallise out again. The fourth crop so obtained had hydrogen absorption 93–94 %. When a hydrogen absorption about 95 % is reached, crystallisation is continued from stronger solutions (1:0·75). When material having hydrogen absorption over 99 % is first reached, the yields of the three best fractions are approximately as follows:

Hydrogen absorption, %	96 to 97	99·3	99·7
Yield, % of starting material	22·7	4·1	2·4

Purification of cinchonine by crystallisation of the dihydrobromide is slow and tedious and, in the hope of expediting the work, other derivatives were tried including the silver nitrate and the zinc chloride compounds, but neither of these was found effective as a means of eliminating dihydrocinchonine.

Cinchonine sulphate argentonitrate was prepared by adding silver nitrate solution (1 ml. of 50 %) to cinchonine sulphate (1 g. in 26 ml. water). On standing overnight a crop of colourless needles separated; wt. 0·984 g. This, on boiling with water (15 ml.), showed slight decomposition, but on filtration through charcoal gave a clear, faintly brown solution, which on cooling deposited nearly colourless needles (0·4 g.) containing $2H_2O$. (Loss at 130° *in vacuo* 4·26: calc. 4·27 %.) M.P. 199° (decomp.). (Found: C, 51·38; H, 6·01; N, 7·82; Ag, 12·88; S, 3·87 %. $(C_{19}H_{22}ON_2)_2.H_2SO_4, AgNO_3$ requires: C, 53·2; H, 5·41; N, 8·18; Ag, 12·5; S, 3·74 %.) As in the case of quinidine sulphate argentonitrate (p. 434), the carbon determinations were persistently low in the recrystallised material, owing no doubt to the slight decomposition already referred to.

Cinchonine zincichlorides. When a zinc chloride solution (4 ml. of 50 % in water) is added to cinchonine sulphate (1 g. in 26 ml. water) an oil is precipitated, which dissolves on addition of alcohol (10 ml.). On standing, a crop (0·9 g.) of colourless needles separates, which from solution in boiling water (20 ml.) deposits on cooling a crop of cinchonine hydrochloride. The mother-liquor on concentration in a vacuous desiccator deposits a *cinchonine hydrochloride zincichloride* represented by the formula $C_{19}H_{22}ON_2.HCl, ZnCl_2$. It can be recrystallised from boiling water or 50 % alcohol. M.P. 282° (decomp.). (Found: C, 48·69; H, 5·13; N, 6·39; Cl, 22·48; Zn, 14·4 %. Calc.: C, 48·8; H, 4·98; N, 6·0; Cl, 22·7; Zn, 14·0 %.) On solution in conc. HCl this substance is converted into a *zincichloride* of the usual type, $C_{19}H_{22}ON_2.2HCl, ZnCl_2$, which can also be formed in the usual manner by adding zinc chloride (2 g. in 3 ml. conc. HCl) to cinchonine (2 g.) in conc. HCl (10 ml.). This crystallises in anhydrous colourless, hair-like needles, from water, dilute alcohol

or conc. HCl (1 g. in 8 ml.). M.P. 263° (decomp.). (Found: C, 45·5; H, 5·08; N, 5·76; Cl, 28·04; Zn, 12·7 %. Calc.: C, 45·2; H, 4·80; N, 5·56; Cl, 28·1; Zn, 12·9 %.)

Two cinchonine zincichlorides have been described already [Gräzinghoff, 1865] to which the following formulae were assigned $(C_{19}H_{22}ON_2.2HCl)_2, ZnCl_2, 2H_2O$ and $(C_{19}H_{22}ON_2.3HCl)_2, ZnCl_2, H_2O$. It is suggested that these products were probably mixtures in which each of the two zincichlorides referred to above was still contaminated with cinchonine hydrochloride, which in the present instance separated as a first fraction in recrystallising the crude product.

The base recovered from the normal zincichloride made from crude cinchonine showed hydrogen absorption 84·0 % as against 86·2 % for the starting material, so that this derivative is of no value for purification purposes.

Cinchonine cuprichloride. The possibility of using the cuprichloride as a means of purifying cinchonine was investigated as described under quinidine (p. 435). After numerous trials, the best results were obtained by using equal quantities of cinchonine and cupric chloride dissolved separately to produce a final solution of solids (1 g.) in conc. HCl (8 ml.), recrystallising the resulting cuprichloride from hot conc. HCl (1 g. in 3 ml.), and recovering the base in the usual way from the recrystallised cuprichloride. Cinchonine dihydrochloride is very soluble in water so that no further fractionation can be effected by solution of the cuprichloride in a small quantity of water as in the case of quinidine (p. 435). The following are typical results:

	Hydrogen absorption, %			
Initial cinchonine	71·5	85·2	96·4	96 to 97
Recovered cinchonine	89·6	96·4	99·0	100·1
Yield, %	78·0	88·0	61·0	59·4

That is, starting with quinidine-free "cinchonine" showing hydrogen absorption of about 85 %, two passages through the cuprichloride are necessary to raise the hydrogen absorption to 99–100 %.

The cinchonine used for the final determination of the constants recorded in Table IV was prepared partly by fractionation of the dihydrobromide and partly by the cuprichloride method, and no difference could be detected between the two products.

Table IV. *Constants of pure cinchona alkaloids.*

Methoxyl-free bases: cinchonidine, dihydrocinchonidine; cinchonine, dihydrocinchonine.

Substance	α_D	c*	Solvent	$[\alpha]_D^{15°}$ calc. for Salt	$[\alpha]_D^{15°}$ calc. for Base	Additional constants
Cinchonidine (Cd):						
Base, M.P. 204·5°	− 2·617°	*M*/40	*N*/10 H_2SO_4	—	−178°	H.A.†=101·2
Acid sulphate, $Cd.H_2SO_4, 5H_2O$	−10·498	*M*/10	Water	−133·9°	−178·5	H.A.=100·2: p_H=2·84
,, ,,	− 2·613	*M*/40	,,	−133·6	−178·1	p_H=3·05
,, ,,	− 2·615	*M*/40	*N*/10 H_2SO_4	−133·4	−177·8	—
Dihydrobromide, $Cd.2HBr, 2H_2O$	−10·112	*M*/10	Water	−110·9	−172·0	p_H=2·52
	− 2·607	*M*/40	,,	−114·3	−177·3	p_H=2·89
	− 2·604	*M*/40	*N*/10 H_2SO_4	−114·2	−177·2	—
Dihydrocinchonidine (HCd):						
Base, M.P. 232°	− 2·140	*M*/40	*N*/10 H_2SO_4	—	−144·6	—
Acid sulphate, $HCd.H_2SO_4, 5H_2O$	− 8·417	*M*/10	Water	−106·8	−142·2	—
,, ,,	− 2·132	*M*/40	*N*/10 H_2SO_4	−108·3	−144·1	—
Dihydrobromide, $HCd.2HBr, 2H_2O$	− 8·149	*M*/10	Water	− 89·0	−137·7	—
,, ,,	− 2·128	*M*/40	,,	− 92·9	−143·7	—
Cinchonine (Cn):						
Base, M.P. 254°	+ 3·876	*M*/40	*N*/10 H_2SO_4	—	+263·7	H.A.=100·1 %
Dihydrobromide, $Cn.2HBr$	+15·238	*M*/10	Water	+167	+259·2	H.A.=99·74 %
	+ 3·867	*M*/40	,,	+169·6	+263·1	—
Dihydrocinchonine (HCn):						
Base, M.P. 267°	+ 3·342	*M*/40	*N*/10 H_2SO_4	—	+225·8	—
Dihydrobromide, $HCn.2HBr$	+13·154	*M*/10	Water	+143·6	+222·2	—
,, ,,	+ 3·347	*M*/40	,,	+146·2	+226·1	—

* c is expressed on dry substance.
† H.A. = hydrogen absorption: % of calculated value (p. 432).

Dihydrocinchonine. The base prepared by catalytic hydrogenation of pure cinchonine was finally purified by crystallisation from boiling alcohol, and from this the dihydrobromide was prepared in the usual way. Dihydrocinchonine differs from the three other dihydro-cinchona bases dealt with in this paper by decolorising permanganate in the standard test (p. 432) in 30 seconds. The *cuprichloride* was prepared by adding the base dissolved in conc. HCl (1 g. in 2 ml.) to cupric chloride in the same solvent (1 g. in 3 ml.). The yield was 0·9 g., which was reduced to 0·45 g. by recrystallisation from 1 ml. hot conc. HCl. From hot concentrated solutions it separates at once in golden-brown needles, but if crystallised slowly, it forms transparent much-twinned garnet-coloured prisms M.P. 212° (both forms). (Found: C, 45·20; H, 5·25; N, 5·54; Cl, 28·21; Cu, 12·31 %. $C_{19}H_{24}ON_2.2HCl$, $CuCl_2$ requires: C, 45·27; H, 5·16; N, 5·56; Cl, 28·16; Cu. 12·62 %.)

The constants found for the base and dihydrobromide are given in Table IV.

Biological tests[1].

Antimalarial activity. The comparison of the activity of the alkaloids was made with canaries infected with a strain of bird malaria kindly supplied by Sir Rickard Christophers. A mixture of the blood from infected birds diluted with 5–10 parts of saline, containing sodium citrate to prevent clotting, was injected under the pectoral muscles of a number of normal birds. The infected birds were bled by cutting off their heads and the feathers excluded by filtering the blood through glass wool. The blood and saline mixture was kept warmed at 37° during the period of the injections, as it was found that the latent period before the appearance of the parasites was considerably longer for the birds which were inoculated last, if this were not done. In the case of warmed blood the latent periods of the birds inoculated first and last do not differ by more than a day if all the injections can be completed in 15–25 minutes. To obviate any systematic error on this score the birds were arranged so that the average time of standing of the inoculum was constant for each group of birds used for the different drugs. The drugs were given orally by the method of Roehl through a thin piece of rubber tubing. The first dose was given 4 hours after the injection of the infecting material. Subsequent doses were given at daily intervals for the next 5 days, making 6 doses in all. The drugs were given in proportion to the weight of the bird, in the majority of cases, 5 or 2·5 mg. of base dissolved in 0·5 ml. per 20 g. weight of bird (most of the birds weighed 13–18 g.). The birds tolerate the material better if it is injected slowly. Six birds were usually used for each drug and the potency was estimated in comparison with that of quinine by taking the average time after infection at which parasites appeared in a particular group and comparing it with the average of the quinine group done at the same time. The authors consider this point of importance in the comparison of the activity of the drugs. The average time of appearance of parasites after inoculation and treatment with 6×5 mg. doses of quinine has varied from 16 to 23 days in these and other experiments not yet recorded. These differences show that unless in each batch of infected birds a group is treated with some drug to serve as a standard of curative activity, quantitative estimations of relative activity will suffer from a larger and almost incalculable error. The error of such comparisons has been very roughly calculated, and a preliminary estimate suggests that with two groups of six birds a difference in mean time of appearance of parasites of 1 day is significant and for two groups of three birds, a difference of 1·5 to 2 days.

Occasionally it has not been possible to detect parasites in treated birds for 30 days after the infection. These birds have been reinjected with infected blood

[1] G. A. H. Buttle and J. W. Trevan are responsible for all the experiments on animals.

and if they have shown parasites after the infection, they are considered to have been complete cures.

The results are shown in Tables C and D.

Table C is a typical group treated with quinine. It shows the differences obtained in a group of birds treated in the same manner.

Table D shows the results obtained in different experiments.

Table C.

Bird No.	Drug	Proportion of red cells containing parasites (days after inoculation)						
		17	18	19	20	21	22	25
45	Quinine				(Died 5th day)			
46	6 × 5 mg.	—	VVF	—	1/15	—	1/50	—
47		—	—	—	—	—	—	—
48		—	VVF	—	1/15	—	1/15	—
49		—	VVF	—	1/10	—	1/10	—
50		—	—	—	1/120	1/100	—	1/40

VVF indicates one or two parasites in several fields.

Table D.

Date	Control. Average time of appearance of parasites in untreated birds (in days)	Drug (6 doses in each case)	Dose mg. per 20 g. bird weight	Average time of appearance of parasites in treated birds (in days)
13. i. 31	7·0	Dihydroquinine	5	21·6 + 2 cures
		Quinine	5	18·2 + 1 cure
		Dihydroquinidine	5	15·6
		Cinchonidine	5	14·0
		Dihydrocinchonine	5	11·0
		Dihydrocinchonidine	5	9·3
2. ii. 31	5·9	*Quinine*	5	17·6
		Dihydroquinine	3·5	16·3
		Dihydroquinidine	5	16·0
		Cinchonidine	5	14·6
		Quinine	2·5	14·6
		Quinine	1	14·0
		Dihydrocinchonine	5	12·0 (1st dose 10 mg.)
		Dihydrocinchonidine	5	11·2 (1st dose 10 mg.)
18. iii. 31	4·0	*Quinine*	5	16·0
		Quinidine	5	14·0* (2 birds only)
		Cinchonine	5	12·3
7. iv. 31	5·6	*Quinine*	5	19·0* (1 bird only)
		Quinidine	5	17·0* (1 bird only)
		Cinchonine	5	13·0

* Six birds were used for each group, usually some died during the test; if there were less than three survivors, it has been noted in the last column.

Toxicity. The toxicity of certain of these substances on intravenous injection was examined by intravenous injection into mice. The relevant results are given in Table E.

The average lethal doses are recorded in the last column. The figures represent the estimate of the dose necessary to kill 50 % of the mice. The differences between the different alkaloids are probably not statistically significant, with the possible exception of dihydroquinidine.

Table E. *Results of toxicity tests by intravenous injection into mice.*

Drug	Dose in mg. of base per 20 g. mouse	Mortality	Estimate of average lethal dose of base for 20 g.mouse mg.
Quinine acid sulphate	1·2	0/10	1·9
	1·8	4/10	
	2·1	8/10	
	2·4	23/25	
Quinine dihydrochloride	1·05	1/10	1·4
	1·5	6/10	
	1·9	5/5	
	2·25	10/10	
Dihydroquinine acid sulphate	1·0	1/10	1·9
	1·25	0/10	
	1·5	0/10	
	2·0	11/20	
	2·5	5/5	
	3·0	5/5	
Quinidine dihydrochloride	1·0	0/10	1·6
	1·5	5/10	
	2·0	11/15	
Dihydroquinidine acid sulphate	1·0	6/15	1·3
	1·25	2/10	
	1·5	7/10	
Cinchonine acid sulphate	1·0	1/10	1·75
	1·5	2/10	
	2·0	7/10	
Dihydrocinchonine acid sulphate	1·26	0/10	2·0
	1·9	0/10	
	2·2	10/10	
Cinchonidine acid sulphate	1·5	0/10	1·85
	1·75	3/10	
	2·0	17/20	
Dihydrocinchonidine acid sulphate	1·9	0/10	2·0
	2·2	10/10	
Cinchonine chloride hydrochloride	1·3	0/10	1·55
	1·6	7/10	
	1·8	9/10	

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LXII. A MACRO-METHOD FOR THE DETERMINATION OF IRON IN BIOLOGICAL MATERIAL BY A MODIFIED PERMANGANATE TITRATION.

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In the course of investigations on the metabolism of iron, it became necessary to determine relatively large amounts of iron in the presence of such interfering agents as organic matter and phosphate. It was found that the errors introduced by colorimetric micro-methods became enormously exaggerated when amounts of iron between 1 and 10 mg., or more, were determined. A survey of the existing methods which could be used for such amounts of iron showed many disadvantages.

Destruction of organic matter by ignition was extremely time-consuming; incineration with sulphuric acid was considered to present the advantages both of quickness and of removal of halogens which would interfere in subsequent steps in the procedure.

The gravimetric estimation of iron required either the removal of phosphate by molybdate, or the use of the basic acetate separation. These modifications, together with the general undesirability of using ammonia in the laboratory, ruled out this method of investigation.

The use of a sulphuric acid digestion made possible the volumetric estimation of iron. The permanganate method of Margueritte [Treadwell and Hall, 1924] was chosen because of the ease of determining the end-point and the fact that permanganate offers a self-contained indicator. The iron for this method must be in the ferrous condition. This was assured by reduction by nascent hydrogen in the presence of an excess of zinc and sulphuric acid.

The method has proved to be very satisfactory and extremely accurate within the limits of its application. It has been used in some 400 estimations of iron during the past year with uniform success. The results obtained by the method will be published elsewhere, the present paper giving the details of the modifications employed, the precautions and a few examples of its accuracy and possible applications.

Experimental.

Reagents.

Sulphuric acid. "Baker's Analysed," "C.P." brand, low in heavy metals.

Perchloric acid. Merck's brand 60 % perchloric acid, sp. gr. 1·54, low in heavy metals.

Zinc. "Baker's Analysed" zinc, "C.P." brand, 30 mesh granular, low in lead and iron.

Potassium permanganate N/10 and N/100. Standardised according to Treadwell and Hall [1924] against pure sodium oxalate.

Glassware. All glassware was of pyrex, extracted with 2 % HCl, chromic acid and rinsed in many changes of distilled water. The distilled water should be checked for the presence of iron and if necessary redistilled from treated glass vessels.

Destruction of organic matter.

(*a*) *Solutions of soluble iron compounds.* An amount of the iron solution containing 5–10 mg. of iron was measured into an 800 ml. Kjeldahl flask. 10 ml. of concentrated sulphuric acid and two glass beads were added and digestion commenced in a Kjeldahl digestion apparatus over a low flame. After charring was complete and a blood-red, apparently homogeneous solution was obtained, 0·5 ml. of perchloric acid was added. Digestion was then continued for one hour. Oxidation was invariably complete at this time and the solution was free from chlorides.

(*b*) *Faeces.* 1 g. samples were used when patients were being treated by some form of iron therapy. When a low iron content of the faeces necessitated the use of much larger samples than this, it was deemed advisable to use one of the many micro-methods. The faeces were prepared for analysis by careful drying, first on the water-bath and later in a desiccator. The sample should be kept as finely divided as possible during the drying process. The sample for analysis was further finely ground and sampled by quartering. 1 g. samples of this finely ground material were transferred directly to an 800 ml. Kjeldahl flask and completely washed into the flask by distilled water. 10 ml. of sulphuric acid and two glass beads were added. The digestion was allowed to proceed very slowly, the flasks being rotated from time to time until homogeneous mixtures, free from foam, were obtained. At this point the speed of digestion was increased until golden-red, translucent solutions resulted. 0·5 ml. of perchloric acid was then added and the digestion continued for a further hour.

(*c*) *Whole blood.* 2–10 ml. of whole blood were used for the analysis, depending on the degree of anaemia. The digestion was carried out in the same way as for faeces. (To facilitate digestion the Kjeldahl flasks containing the blood and sulphuric acid may be allowed to stand overnight before the digestion commences.)

(*d*) *Insoluble iron compounds.* Insoluble iron compounds were analysed in the same way as for faeces. The amount taken for analysis however should contain at least 5 mg. of iron as the metal.

Reduction.

The contents of the Kjeldahl flasks were diluted with 15 ml. of water and raised to the boiling-point with constant rotation. The sulphuric acid solution was then transferred quantitatively to 125 ml. Erlenmeyer flasks. 10 ml. of distilled water were added to the Kjeldahl flasks, boiled and transferred quantitatively to the Erlenmeyer flasks. The volume of transfer fluid should be kept as low as possible. Boiling out the Kjeldahl flasks was found to be absolutely necessary since, even with the greatest care, some etching of the glass occurs.

The contents of the Erlenmeyer flasks were allowed to cool completely, and 5 g. of granulated zinc were added. The flasks were then fitted with Bunsen valves and allowed to stand overnight.

When the reaction between the zinc and sulphuric acid was complete, a small sample of the contents of the flask was removed by means of a capillary tube and the fraction of a drop so removed tested for ferric iron with ammonium thiocyanate, using a porcelain spot plate.

Titration.

When reduction was complete the contents of the Erlenmeyer flasks were filtered quantitatively through No. 42 Whatman filter-papers. The flasks were washed by decantation and the final residue transferred by a stream of water to the filter-paper. The filtrate, including the washing, was collected in 400 ml. pyrex beakers. Washing and transferring were done in the cold as quickly as possible to avoid re-oxidation of the iron.

2 ml. of concentrated sulphuric acid were added to the contents of the beakers and titration with 0·1 *N* potassium permanganate carried out. During the titration the beakers were surrounded by a white background and the end-point taken as the first recognisable pink flush persisting for 30 seconds.

When the amount of iron was less than 10 mg. it was found advisable to use 0·01 *N* potassium permanganate. In this case all transfers from the beginning of the procedure should be made with the minimum amount of wash fluid. To diminish further the dilution of the iron, the final transfers and washings may be made with cold 1 % sulphuric acid. The addition of further sulphuric acid to the solution before titration may then be omitted. The final titration was done in white porcelain casseroles which facilitated identification of the end-point with certainty. It was found advisable to use a standard end-point at first, but when the eye became accustomed to the recognition of the end-point, this precaution was omitted. All titrations were done with a Folin micro-sugar burette of 5 ml. capacity, graduated in 0·02 ml. to facilitate splitting of drops.

Blank.

A blank was run on all reagents by carrying through the exact procedure without the addition of the iron-containing material. This blank was subtracted from the total titration figure.

Precautions.

The oxidation of organic matter and the removal of interfering agents by sulphuric acid digestion was found to be straightforward if the directions were closely followed. Time was saved by digesting as slowly as possible at first and only increasing the temperature after all danger of foaming was over.

Much time was spent in determining a satisfactory form of zinc to employ in reduction. The 30-mesh zinc suggested was found to be the right size to prevent floating, if added after the solutions to be reduced were cool. 5 g. of zinc are more than adequate for the usual amounts of iron handled and this amount of zinc will keep the iron in a reduced condition for 12 hours, if the Bunsen valves are made air-tight.

From the time the reducing flasks are opened, no delay should be made. Re-oxidation of the iron was found to be quite rapid; however, if the titration follows the transfer within 15 minutes, no appreciable change in the titre can be found. In order to reduce the time still further, at this point, filtration may be made through platinum cones if such are available.

Experimental results.

The method has been used for the analysis of iron in such compounds as ferric ammonium citrate, in the presence of organic matter, phosphates and chlorides. It has also been used in the estimation of iron in such biological

material as faeces and blood. The data presented in Tables I and II indicate the degree of duplication and accuracy which has been in general obtained by the method.

Table I. *Recovery of added iron from dried faeces.*

Weight of faeces g.	Iron content of faeces mg.	Iron added mg.	Total iron found mg.	Iron recovered mg.	Error mg.
1·000	9·90	9·98	19·95	10·05	+0·07
1·000	9·90	9·98	19·85	9·95	−0·03
1·000	9·90	9·98	19·85	9·95	−0·03
1·000	9·90	5·00	14·80	4·90	−0·10
1·000	9·90	5·00	14·83	4·93	−0·07

Table II. *Analyses of 1 % and 0·1 % ferric ammonium citrate solution containing iron-free organic matter*.*

ml. of solution	%	Iron present mg.	Iron found A mg.	Iron found B mg.	Iron found Average mg.	Error mg.
50	1	88·95†	88·95	88·95	88·95	±0·00
25	1	44·47	44·35	44·25	44·30	−0·17
10	1	17·79	18·15	18·05	18·10	+0·31
5	1	8·89	9·05	9·15	9·10	+0·20
50	0·1	8·89	8·60	8·55	8·57	−0·32
25	0·1	4·45	4·21	4·27	4·24	−0·21
10	0·1	1·78	1·70	1·71	1·705	−0·07
5	0·1	0·89	0·80	0·62	0·71	−0·18

* Filter-paper. † Calculated from repeated estimations of ferric ammonium citrate.

In Table I recovery of added amounts of a standard iron solution (ferric chloride prepared from pure iron wire) from faeces is given. For amounts of iron above 5 mg., recoveries to within 0·1 mg. were obtained. The duplication of results was good; variations between duplicates of 0·03 mg. of iron were found. The iron values for the solutions given were obtained by the customary gravimetric method.

Table II presents data obtained from the analysis of standard solutions of ferric ammonium citrate. This compound was used in the investigation for which this method was devised and represents iron attached to an organic radical. Further organic matter was added in the form of filter-paper during the digestion. The error in mg. varied from 0·07 to 0·32 throughout the range of dilutions. The amount of iron present varied from 0·89 to 88 mg.

Table III is included as an example of the use of the method for general biological analyses. The patients had varying degrees of anaemia. The haemoglobin value was determined in two cases by direct estimation from the oxygen capacity and in the third, by use of the Sahli haemoglobinometer standardised against the oxygen capacity. The volumes of blood taken varied from 2 to 10 ml.

Table III. *Determination of the iron content of haemoglobin.*

Patient	ml. blood	Iron mg./100 ml. blood	O_2-capacity vol./100 ml. blood	Hb g./100 ml. blood	% iron in Hb
1	6	53·2	19·95	14·82	0·359
2	10	38·2	—	11·39*	0·345
3	2	45·8	17·91	13·30	0·342

* Sahli.

The determined percentage of iron in haemoglobin found in all cases checked to within 0·02 %. The values found for the percentage of iron in haemoglobin were slightly higher than those calculated from the figures of Murphy *et al.* [1931], who used a micro-method, but in close agreement with those of Hufner [1894]. From the duplication of results it appears that the method lends itself very well to whole blood determinations.

Reports by Brock and Taylor [1934] show that the method may be satisfactorily used in dialysis and metabolic studies of iron, when this metal is present in gross amounts.

SUMMARY.

1. A detailed description is given of a modification of the permanganate titration method for the determination of iron.

2. The method is applicable to the determination of iron in the presence of organic material and phosphate.

3. Data are presented showing the degree of accuracy and duplication of the results.

4. The satisfactory use of the method in the determination of the percentage of iron in haemoglobin is demonstrated.

5. The method is recommended for the determination of iron in all biological material in which samples can be obtained containing more than 2 mg. of iron per g.

6. Within the limits specified for its application, iron may be determined within $\pm 0{\cdot}3$ mg.

One of us (J. F. B.) was Leverhulme Research Scholar of the Royal College of Physicians of London at the time that the work was done, and expresses his appreciation to the Science Committee of the College for the privilege of spending the year in Boston.

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LXIII. THE DIFFUSION OF SOLUBLE IRON COMPOUNDS *IN VITRO*. THE EFFECT OF ACIDS, BASES AND ELECTROLYTES.

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Although Blaud, when in 1831 he introduced his iron pill for the treatment of chlorosis, recognised the need for administering iron in large doses, this principle was largely overlooked until recent years, when it has been revived in the treatment of hypochromic anaemia. It is recognised to-day that adequate oral iron therapy requires considerably larger quantities than were employed ten years ago.

It has always been appreciated, nevertheless, that much of the iron administered by mouth is unabsorbed and excreted in the faeces. Recently it has been shown by Heath *et al.* [1932] that in idiopathic hypochromic anaemia at low levels of haemoglobin, parenterally administered iron in the form of iron citrate is converted approximately quantitatively into haemoglobin. This observation resolves the problem of adequate oral iron therapy into a problem of the absorption of iron from the gastro-intestinal tract.

To raise the haemoglobin level of an adult by 2 % per day, which can readily be achieved in uncomplicated cases by adequate oral iron therapy, requires the absorption from the gastrointestinal tract of approximately 50 mg.[1] of metallic iron per day. The introduction of that amount of iron daily by injection of any existing iron compound intended for parenteral administration is, for the patient, always an uncomfortable and sometimes a dangerous procedure. It is obvious therefore that there is as yet no adequate substitute for the oral administration of iron. Very few disadvantages have been demonstrated in the oral administration of large doses of iron, and in every way this form of therapy is advisable in hypochromic anaemia.

A study of absorption from the gastro-intestinal tract cannot be made, of course, *in vitro*, but there are certain physicochemical relationships which may play a part in gastro-intestinal absorption which can be so studied, *e.g.* the chemical reactions which will lead to the presence of iron in a soluble form in the gastro-intestinal tract and the effect of such conditions as the hydrogen ion concentration on these reactions and on the rate of dialysis of iron.

The object of the present investigation was to determine which forms of soluble iron most readily dialyse through cellophane membranes and what conditions affect the rate of dialysis.

[1] Taking 5 litres as an average figure for adult blood volume, 15·6 g. per 100 ml. as 100 % haemoglobin and 0·34 % as the iron content of haemoglobin.

Methods.

Complete assembly of the apparatus used for the series of dialysis experiments is shown in Fig. 1. Seamless cellophane tubing[1] was found to be the most satisfactory medium for dialysis. The tubing was cut into suitable lengths and soaked for one hour in distilled water to remove the glycerol preservative. Each section of tubing was made into a sac by double tying and sealing with

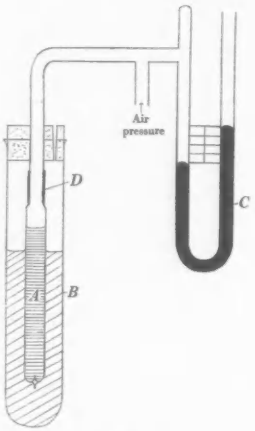

Fig. 1.

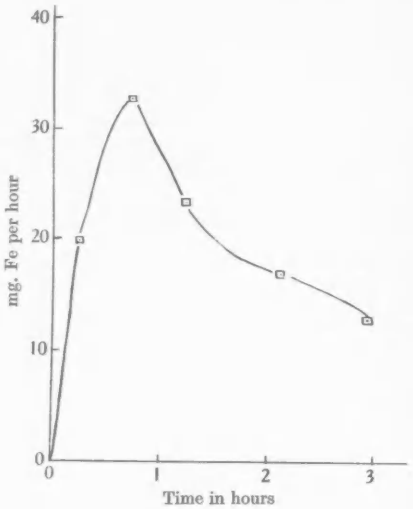

Fig. 2.

Fig. 1. Diagram of apparatus. *A*, cellophane sac containing iron mixture. *B*, diffusion medium. *C*, mercury manometer—20 mm. Hg. *D*, wax sealing cellophane on to glass tubing.

Fig. 2. Typical rate curve. Ferric ammonium citrate against serum.

collodion. Frequent checking throughout the study demonstrated that there was no leakage, and duplication of results was extraordinarily constant, varying by less than 4 % throughout the investigation. These cellophane sacs were sealed by means of paraffin wax, which made air-tight junctions, on to the ends of glass tubes projecting through rubber stoppers. The sacs were tested with applied pressure up to 40 mm. Hg. The iron solution to be studied was introduced into the sac and the diffusion medium into the outside container. Iron was subsequently determined in both the sac contents and the diffusion medium by means of a development of the Margueritte method for the volumetric determination of iron [Treadwell and Hall, 1924; Taylor and Brock, 1934]. By comparing the amount of iron recovered from the sac and from the diffusion medium at the end of the experiment with the amount introduced initially into the sac, it was possible to check accurately the entire procedure so that experimental errors were practically eliminated.

[1] Obtained from the Dupont Cellophane Company, 350, Fifth Avenue, New York, and having the following specifications: inflated diameter $\frac{3}{8}$ in., flat width 0·59 in., thickness 0·0006 in.

In order to find the optimum time for iron diffusion, the following experiment was devised. 4 ml. of a 10 % aqueous solution of ferric ammonium citrate were introduced into each of 5 sacs and diffused against normal horse-serum under an applied pressure of 20 mm. Hg. A sac was removed at the end of $\frac{1}{2}$, 1, $1\frac{1}{2}$, 3 and 12 hours. The rate of diffusion at each of these time intervals in mg. iron per hour was determined. These values were averaged for the time interval concerned and plotted against time. A typical curve is shown in Fig. 2. From this experiment it was observed that the curve of diffusion definitely flattens after 3 hours.

In all the experiments a constant positive pressure of 20 mm. Hg was applied to the inside of the sacs. Three different diffusion media were employed during the investigation, normal horse-serum, a special diffusion medium and distilled water. The special diffusion medium referred to in the text as "special saline," was introduced to avoid a protein precipitation which sometimes occurred with serum. It is essentially a protein-free blood-serum. The composition was based on the determination of the constituents of normal human serum as given by Mathews [1930]. 2 litres of solutions contained

Potassium sulphate	0·584 g.
Potassium chloride	0·746 ,,
Sodium chloride	11·520 ,,
Disodium hydrogen phosphate	0·564 ,,
Sodium bicarbonate	3·200 ,,
Calcium hydrogen phosphate	0·620 ,,
Magnesium chloride	0·464 ,,

The rate of diffusion was determined of a 10 % aqueous solution of ferric ammonium citrate into each of the three diffusion media.

Experimental.

First, the effect of various conditions on the rate of diffusion of ferric ammonium citrate was studied. Secondly observations were made on the rate of diffusion of some other soluble iron compounds, and the results were compared with those of ferric ammonium citrate. All experiments were performed in duplicate.

(1) *The effect of increasing concentration on the rate of diffusion of ferric ammonium citrate.*

5, 10 and 20 % aqueous solutions of ferric ammonium citrate were dialysed against normal horse-serum for the following periods: $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 3 hours. In each case 4 ml. of the solution were introduced into the sac and 20 ml. of normal horse-serum into the container. The results are shown in Fig. 3, in which the percentage diffused was plotted against time. It will be observed that the three curves practically coincide. When these results are plotted in the form of an absolute rate curve, Fig. 4, it will be observed that the amount passing through the membrane in a given time is directly proportional to the concentration within the sac.

(2) *The effect of acid and alkali on the rate of diffusion of ferric ammonium citrate.*

10 % solutions of ferric ammonium citrate were made up in distilled water, 0·1 *N* HCl and 0·1 *N* NaOH. 4 ml. of each of these solutions were dialysed against 20 ml. of normal horse-serum for $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 3 hours. The p_H of the

solutions varied from 1·5 in the case of the acid iron solution to 12·0 in the case of the alkaline iron solution. The results are given in Fig. 5.

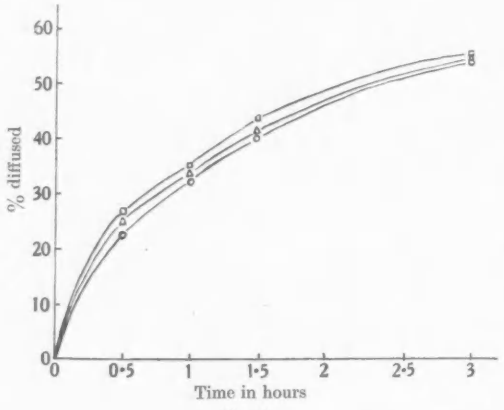

Fig. 3.

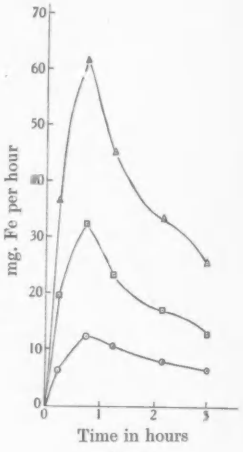

Fig. 4.

Fig. 3. Effect of concentration on the rate of diffusion of ferric ammonium citrate into serum. □ 20 %; △ 10 %; ○ 5 %.

Fig. 4. Rate curves. △ 20 % ferric ammonium citrate; □ 10 % ferric ammonium citrate; ○ 5 % ferric ammonium citrate.

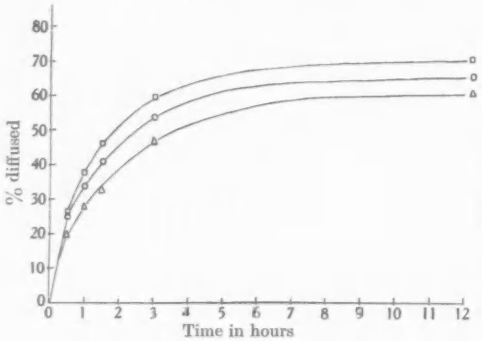

Fig. 5.

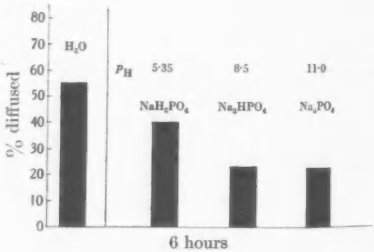

Fig. 6.

Fig. 5. Effect of acid and alkali on the diffusion rate of ferric ammonium citrate into serum. □ 0·1 *N* HCl; ○ H_2O; △ 0·1 *N* NaOH.

Fig. 6. Effect of 0·7 *M* phosphate ion on the diffusion of ferric ammonium citrate into serum.

The figure shows that the rate of diffusion from an acid medium is slightly faster than that from distilled water and the rate from an alkaline medium is slightly slower.

(3) *The effect of sodium phosphate on the rate of diffusion of ferric ammonium citrate.*

Sufficient Na_2HPO_4 was added to an aqueous solution of ferric ammonium citrate to make solutions containing 10 % ferric ammonium citrate which were respectively 0·035, 0·07, 0·35 and 0·7 *M* with respect to Na_2HPO_4. Similar solutions were made using NaH_2PO_4 and Na_3PO_4. 4 ml. of each of these 12 solutions were dialysed against 20 ml. of normal horse-serum for 6 hours. The results for the 0·7 *M* phosphate solutions are given in Fig. 6. In the case of NaH_2PO_4, the p_H of which was 5·35, there was a slight inhibition of the rate of dialysis as compared with a simple 10 % aqueous solution of the ferric ammonium citrate. In the case however of the two alkaline phosphates, the p_H values of which were 8·5 and 11·0, the inhibition of diffusion was very considerable. No precipitate whatsoever could be detected in any of these solutions. From a study of the other phosphate concentrations, it was found that the effect of the phosphate ion decreased with decreasing concentration. In the case of NaH_2PO_4, the inhibition of the rate of diffusion of the iron salt was of the same order as that found for neutral sodium chloride. For the alkaline phosphates, however, the depression of the rate of diffusion by the higher concentrations was approximately twice that of neutral sodium chloride.

The absolute rate of diffusion of ferric ammonium citrate in 0·7 *M* Na_2HPO_4 is plotted against time in Fig. 7. Special saline was used as the diffusion medium. It will be observed that there was almost complete inhibition of the diffusion of ferric ammonium citrate and that what diffusion did occur, proceeded at a constant rate. Since no precipitate was found during the experiment, it was necessary to consider the possibility that the iron and phosphate together were modifying the capillary permeability of the cellophane membrane by forming a true adsorption compound within the pores of the membrane. Such phenomena occur when copper sulphate and potassium ferrocyanide are independently diffused through porcelain membranes, resulting in a complete change in the character of the membrane. Such adsorption compounds are usually permanently fixed in the membrane. In order to test this possibility, 10 % aqueous solutions of ferric ammonium citrate were diffused for 1·5 hours into normal horse-serum. The sacs were then washed out thoroughly in distilled water, and a 10 % solution of ferric ammonium citrate in 0·7 *M* Na_2HPO_4 was diffused through the same membrane for 1·5 hours. The sac was once more thoroughly washed out in distilled water, and a 10 % solution of ferric ammonium citrate was diffused again through the membrane. The results are given in Fig. 8 and show that the rate of diffusion of the ferric ammonium citrate through the membrane was exactly the same after the passage of iron and phosphate together as it was before.

(4) *The effect of some other electrolytes on the diffusion of ferric ammonium citrate.*

The effect of some other electrolytes on the rate of diffusion of ferric ammonium citrate was studied. 10 % solutions of these substances—ammonium chloride, sodium citrate, sodium bicarbonate and sodium chloride—were used, in which ferric ammonium citrate had been dissolved to give also a concentration of 10 % with respect to the iron salt. 4 ml. of each of these solutions were then diffused against 20 ml. of special saline for $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 3 hours. In this experiment special saline was preferred to serum in order to avoid the

occasional precipitation of serum-proteins by the electrolytes. The results are shown in Fig. 9, in comparison with a block for the diffusion of 10 % ferric ammonium citrate in distilled water under the same conditions. These various electrolytes decreased the rate of iron diffusion at the 3-hour period by amounts varying from 8 % for ammonium chloride and potassium citrate to 18 % for

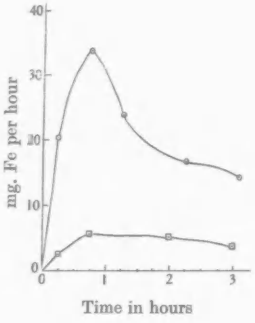

Fig. 7.

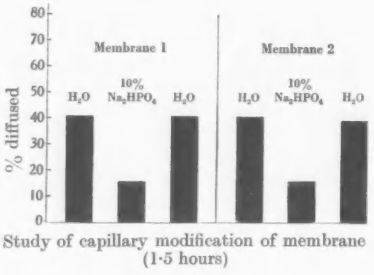

Fig. 8.

Fig. 7. Effect of phosphate. ○ Ferric ammonium citrate in H_2O; □ Ferric ammonium citrate in 0·7 *M* Na_2HPO_4.

Fig. 8. Showing the failure of ferric ammonium citrate in the presence of phosphate ion to change the characteristics of cellophane membranes.

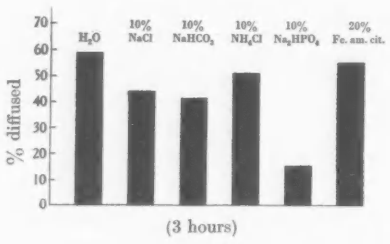

Fig. 9.

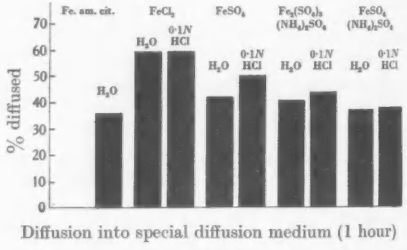

Fig. 10.

Fig. 9. Effect of various electrolytes on the diffusion of ferric ammonium citrate into special diffusion medium.

Fig. 10. Effect of acid on the diffusion of some iron salts.

sodium bicarbonate. There seems to be no relationship between the action of those electrolytes which are acid on hydrolysis and of those which react as bases. In spite of the common citrate ion, neither potassium nor sodium citrate decreased the rate of iron diffusion below that found for sodium chloride. Fig. 9 demonstrates clearly the striking inhibitory action of the alkaline phosphates on the diffusion of ferric ammonium citrate, while Fig. 6 demonstrates that the acid sodium phosphate has a much weaker inhibitory effect, which is of the same order as those of the other electrolytes tested.

(5) *A comparison between the diffusion of ferric ammonium citrate and that of some other soluble iron compounds.*

The rates of diffusion of ferric chloride, ferrous sulphate, ferrous ammonium sulphate and ferric ammonium sulphate were studied under conditions comparable with those used in the case of ferric ammonium citrate. 4 ml. of a 10 % solution of ferric ammonium citrate, which were used in that study, contained 70 mg. of metallic iron. Therefore, concentrations of the above salts were used such that 4 ml. contained 70 mg. of metallic iron. One- and three-hour periods were used in these experiments, and special saline was employed as a diffusion medium. Each of the iron salts was studied both in solution in distilled water and in 0·1 *N* HCl. The results are given in Fig. 10. The rate of diffusion of each of these salts was greater than that of ferric ammonium citrate, and with the exception of ferric chloride, the diffusion rate out of 0·1 *N* HCl was greater than out of distilled water. Ferric chloride, being an extremely acid salt, was naturally enough not affected by the presence of 0·1 *N* HCl.

All four of these soluble iron salts formed insoluble precipitates when they were added in the concentrations used in these experiments to 0·7 *M* Na_2HPO_4.

Discussion.

The data concerning the effect of increasing the concentration of ferric ammonium citrate on the diffusion of this salt need no comment. Two possible explanations can be offered for the effect of acid and alkali. The membrane itself may be modified by the presence of hydrogen or hydroxyl ions in contact with it, or the acid and alkali may interact directly with the ferric ammonium citrate to form substances having a higher or lower diffusion rate. The effect of electrolytes in general was to decrease the rate of diffusion of ferric ammonium citrate to a moderate degree. This observation is explicable on the ground that when electrolytes are present a portion of the osmotic work is being done by the electrolytes, and consequently, during any given time, a smaller number of molecules of ferric ammonium citrate pass through the membrane. Sodium dihydrogen phosphate acted in the same manner as the other electrolytes tested. The alkaline sodium phosphates however behaved quite differently. From the study of the true rate curve of iron diffusion (Fig. 7), it appears that after reaching a very low rate of diffusion the rate continues at a constant level. It was thought at first that adsorption compounds were formed within the membrane so that instead of a cellophane membrane being present, a cellophane membrane impregnated with ferric phosphate resulted. The crucial experiment described above, indicated quite clearly that such was not the case. In the absence of a precipitate the only reasonable explanation that could be offered was that the membrane became loosely plugged with a colloidal ferric phosphate. The fact that the presence of salts having a common citrate ion did not decrease the rate of diffusion below that found for sodium chloride may be considered evidence that ferric ammonium citrate diffuses through cellophane membranes largely as an undissociated molecule. From this study it has appeared that ferric ammonium citrate, which was chosen because it is a freely soluble iron compound and is used widely as a therapeutic agent, is in some ways unique. It is the only one of the five soluble iron compounds studied, which does not form a precipitate in 0·7 *M* Na_2HPO_4. Secondly, it is the only soluble iron salt which is extensively used as a therapeutic agent. The other forms of therapeutic iron in common use for oral administration being ferrum reductum and ferrous carbonate in the form of Blaud's pill.

It is of interest to compare with the above observations, certain results of investigations *in vivo* on the response of the organism to certain forms of orally administered iron. Mettier and Minot [1931] have shown that in hypochromic anaemia the reticulocyte response following oral administration of ferric ammonium citrate is greater when the iron is administered in an acid-buffered medium than when it is given in an alkaline-buffered medium. It was shown by Marlow and Taylor [1934] that the urinary excretion of iron is raised when ferric ammonium citrate is given orally in acid-buffered solutions, but not when it is given in alkaline-buffered solutions. These two observations indicate that ferric ammonium citrate, at any rate, is more readily absorbed when it is administered in an acid-buffered solution. Mettier [1930] found that the acid-buffered beef-steak medium, which he used, very materially altered the p_H of the duodenum for 2–3 hours, the time being longer when achlorhydria was present. The fact that the acid-buffered medium had a more prolonged effect in increasing the hydrogen-ion concentration of the duodenum of achlorhydric individuals than in the case of patients with a normal gastric acidity is interesting, although Mettier makes no comment on it. It appears to indicate that achlorhydric individuals are also deficient in their power to secrete alkali into the duodenum. Macallum [1893] and Abderhalden [1900] have claimed that iron is principally absorbed from the duodenum. Mitchell and Miller [1929] have shown that the ash of spinach, which contains iron, copper and manganese, is a better haemoglobin-building supplement to a milk diet in anaemic rats when the ash is dissolved in hydrochloric acid than when it is mixed in insoluble form with the food paste. It was shown by Cox *et al.* [1931] that soluble aluminium salts and ferric salts, when added to the diet of guinea-pigs in excess of the phosphorus present, cause a marked lowering of the inorganic phosphorus of the blood and of the calcium and phosphorus in the ash. This effect can be prevented by the addition to the diet of sodium dihydrogen phosphate in amount equivalent to the iron or aluminium. They state that these effects are due to the precipitation of alimentary phosphorus as the ferric and aluminium phosphates.

Brock and Diamond [1934], working with rats, have shown that the addition of large amounts of ferric or ferrous salts to a non-rachitogenic diet produces rickets. They have shown that the rickets can be prevented by the addition to this iron-containing diet of sufficient phosphate theoretically to combine with all the iron present. This work was undertaken as a sequel to the observations described in this paper on the effect of phosphates on the dialysis of iron and ammonium citrate.

So little is known about absorption from the gastro-intestinal tract that it is impossible to do more than speculate on the interpretation of some of these observations. But they do indicate the probability that reactions in the gastro-intestinal tract between iron and acids, bases and electrolytes may play an important rôle in determining the availability of that iron for absorption by the organism. Studies *in vitro* of such reactions may therefore lead to knowledge of the conditions favouring the absorption of iron.

Summary.

1. The rate of dialysis of certain soluble iron compounds across cellophane membranes has been studied under various conditions.

2. The rate of dialysis of ferric ammonium citrate is directly proportional to the concentration of the salt.

3. The rate of dialysis of ferric ammonium citrate into serum and into a special non-protein diffusion medium is increased by the presence of 0·1 *N* HCl. It is decreased slightly by 0·1 *N* NaOH, moderately by the presence of a variety of electrolytes, including sodium dihydrogen phosphate, and very markedly by secondary and tertiary sodium phosphates, in the absence of precipitates.

4. The rates of dialysis of certain other soluble iron compounds are compared with that of ferric ammonium citrate.

5. Certain observations *in vivo* recorded in the literature on the response of man and animals to various forms of iron therapy are set out for comparison.

The authors express their appreciation to Dr George R. Minot for his interest and co-operation during the progress of this investigation and to Miss Eleanor R. Shea for painstaking technical assistance.

One of us (J. F. B.) was Leverhulme Research Scholar of the Royal College of Physicians of London during the investigation and expresses his thanks to the Science Committee of the College for the privilege of spending the year in Boston.

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LXIV. A SIMPLE METHOD FOR THE PURIFICATION OF *CITRUS* PECTIN.

By HOMI RUTTONJI NANJI and JAMSHEDJI JIJIBHOY CHINOY.

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The preparation of highly purified pectin has hitherto depended upon the use of electrodialysis, prolonged settling of impurities [*cf.* Emmett, 1926] and similar methods. In any event, it is doubtful whether pure samples of esterified pectin have been obtained; only calcium pectate and pectic acid were regarded as obtainable in a pure state. The development of a method for preparing the esterified (soluble) pectin in a state of purity was, therefore, desirable. It has been found possible to do this readily by removing the impurities usually associated with pectins (prepared from *Citrus* fruits) by a method involving the use of iodine and bromine as oxidising agents in aqueous solution.

Norman and Norris [1930] had observed that pectin is very stable to oxidation with bromine in presence of calcium carbonate. Block and Cowgill [1932] had already used a method of oxidation to remove gums *etc.* from antineuritic vitamin extracts.

Iodine and bromine are usually employed as oxidising agents in alkaline solution. The presence of any alkali is evidently ruled out in this case on account of the extreme susceptibility of the ester groups in pectin to alkalis. However, it was found that oxidation of the impurities readily took place in a neutral or faintly acid medium without any appreciable effect on the pectin. Dilute solution of hydrogen peroxide was also tried without success.

Experimental.

Purification by partial oxidation with halogens.

General method. Pectin solution, about 0·5 %, was prepared by dissolving the pectin in boiling water. To 50 ml. of the cooled pectin solution about 3 ml. of $N/10$ iodine solution were added. The oxidation was allowed to proceed for varying periods and at different temperatures. The excess of iodine was then removed by shaking two or three times with ether until the aqueous layer was colourless. The excess could also be removed by warming *in vacuo* at 40°, or by bubbling air through the solution at the same temperature. Pectin was then precipitated by 95 % alcohol and after two hours filtered on muslin, finally passed through various grades of alcohol and dried in the oven at 100° for 20 hours.

Two commercial varieties of pectin were purified and a sample of grape fruit pectin, obtained by extraction of the rind with $N/75$ HCl, was similarly treated. In each series a control experiment was carried out, in which the pectin was precipitated without treating with iodine. The methoxyl value[1] and calcium pectate value[2] of the purified samples of pectin were determined (Table I).

[1] The MeO values were determined by the micro-Zeisel method [Pregl, 1930].

[2] The calcium pectate values were determined by taking an aliquot portion of the purified pectin dried at 100° for 20 hours, using the method of Carré and Haynes [1922].

Table I. *Purification with iodine.*

Sample of pectin	Time of oxidation	Temperature of oxidation ° C.	Yield of purified pectin g.	MeO %	Calcium pectate %
Series I:					
1. Commercial pectin I	Control	20	0·23	9·6, 9·6	94·9, 94·78
2. ,,	12 hours	20	0·21	10·6, 10·5	100·0, 100·15
3. ,,	20 ,,	20	0·20	11·0, 11·15	100·4, 100·4
4. ,,	48 ,,	20	0·19	11·4, 11·4	100·7, 100·8
5. ,,	72 ,,	20	0·19	11·6, 11·56	100·9, 100·8
6. ,,	½ hour	90–95	0·18	11·1, 11·0	100·5, 100·7
7. ,,	1 ,,	90–95	0·16	10·9, 10·8	100·2, 100·2
8. ,,	1 ,,	40	0·19	11·5, 11·4	100·8, 100·7
9. ,,	2 hours	40	0·18	11·4, 11·28	100·6, 100·38
10. ,,	6 ,,	40	—	10·9, 10·7	100·8, 100·7
Series II:					
1. Commercial pectin II	Control	20	0·22	7·9, 7·9	93·9, 94·0
2. ,,	24 hours	20	—	8·1, 8·2	96·0, 96·5
3. ,,	48 ,,	20	—	9·1, 9·2	100·0, 100·1
4. ,,	5 days	20	0·19	9·3, 9·2	100·4, 100·3
5. ,,	10 ,,	20	0·17	9·5, 9·4	100·5, 100·4
6. ,,	1 hour	40	0·20	9·4, 9·38	100·7, 100·6
7. ,,	2 hours	40	0·19	9·3, 9·3	100·3, 100·1
8. ,,	6 ,,	40	0·18	8·8, 8·5	100·3, 100·4
9. ,,	½ hour	90	0·18	9·1, 9·26	100·4, 100·3
10. ,,	4 hours	90	0·16	8·6, 8·5	100·2, 100·3
Series III*:					
1. Grape fruit† pectin	Control	20	0·22	9·4, 9·4	93·1, 93·2
2. ,,	1 day	20	0·20	11·2, 11·2	100·1, 100·2
3. ,,	2 days	20	0·20	11·3, 11·2	100·7, 100·5
4. ,,	6 ,,	20	0·18	11·3, 11·3	100·7, 100·8
5. ,,	1 hour	40	0·19	11·3, 11·38	100·4, 100·3
6. ,,	2 hours	40	0·17	11·6, 11·5	100·6, 100·6

* It will be recalled that Norman [1928], while attempting to prepare pure pectins from lemons and oranges, always obtained pectin having an apparent methoxyl value 8·5 %, although the real methoxyl content calculated from calcium pectate yield was 11·4. This low methoxyl value, he suggested, was due to admixture with partially de-esterified pectin and other degradation products produced by the action of the acid of the juice. This difficulty is readily overcome by using the rind instead of the entire fruit.

† Prepared by extraction of the grape fruit rind with $N/75$ HCl; MeO and Ca pectate values corrected for ash.

It will be seen that purification was slow at ordinary temperature, but at 40° it was complete in about 1–2 hours. Longer heating or heating at higher temperatures only tended to cause a slight hydrolysis of the methoxyl groups. The yield of oven-dried purified pectin in all cases was over 70 % of the starting material. Whilst the calcium pectate values showed a removal of 5–6 % of the "impurity," the methoxyl values were raised by 10–20 %. This would tend to show that halogens destroyed some pectin with lower methoxyl content. Also, in Series II, the highest methoxyl value obtained was 9·5 %; this was probably because the sample was a mixture of tri- and tetra-methoxypectic acid.

Purification with bromine water.

The purification was carried out exactly as described with iodine (p. 456) using approximately 20 ml. $N/10$ bromine water for each gram of pectin (Table II).

Table II. *Purification with bromine water.*

Sample of pectin	Time of oxidation	Temperature of oxidation ° C.	Yield of purified pectin g.	MeO %	Calcium pectate %
Series I:					
1. Commercial pectin I	Control	20	0·23	9·6, 9·6	94·9, 94·78
2. ,,	24 hours	20	0·21	11·2, 11·2	100·3, 100·4
3. ,,	48 ,,	20	0·20	11·5, 11·45	100·8, 100·9
4. ,,	72 ,,	20	0·20	11·5, 11·6	100·9, 100·9
5. ,,	1 hour	40	—*	11·4, 11·4	100·7, 100·6
6. ,,	2 hours	40	0·18	11·2, 11·2	100·6, 100·4
7. ,,	½ hour	90	—*	10·9, 10·8	100·3, 100·4
8. ,,	1 ,,	90	0·16	10·5, 10·4	100·2, 100·3
Series II:					
1. Commercial pectin II	Control	20	0·22	7·9, 7·9	93·9, 94·0
2. ,,	2 days	20	0·20	9·6, 9·58	100·1, 100·2
3. ,,	4 ,,	20	—*	9·6, 9·5	100·6, 100·8
4. ,,	1 hour	40	—*	9·6, 9·5	100·5, 100·5
5. ,,	2 hours	40	0·18	9·2, 9·1	100·3, 100·4
Series III:					
1. Grape fruit pectin	Control	20	0·22	9·4, 9·4	93·1, 93·2
2. ,,	24 hours	20	—*	11·3, 11·4	100·3, 100·4
3. ,,	48 ,,	20	0·20	11·3, 11·5	100·6, 100·8
4. ,,	1 hour	40	0·18	11·5, 11·4	100·3, 100·4
5. ,,	2 hours	40	0·18	11·5, 11·4	100·5, 100·5

* Not determined.

On comparing the above results with those obtained with iodine, it will be seen that bromine water was even more satisfactory than iodine, for its action was quicker; also, the excess of bromine was more readily removed, and the purified pectin was perfectly white.

Purification with chlorine water.

Attempts were made to carry out the purification by means of chlorine water under conditions similar to those described above, but without success. Partial de-esterification of the methoxyl groups and degradation of the pectin molecule itself were found to occur. Complete degradation occurred in 3 days at room temperature and in 1 hour at 40° with the production of brown gummy substances whose nature has not been ascertained.

Purification with hydrogen peroxide.

1 g. of the pectin dissolved in 200 ml. water was treated with 10 ml. of hydrogen peroxide (20 vols.) for varying periods at room temperature and the pectin was recovered as usual. At ordinary temperature no change in the methoxy land calcium pectate values was observed (this fact is in conformity with the observations of Norman and Norris [1930]). After treatment at higher temperatures, however, the pectin was completely oxidised, and no precipitate was obtained on addition of alcohol. The nature of these oxidation products is under investigation.

Action of halogens on sodium pectate and pectic acid.

As the halogens seemed to have slight action on partly de-esterified pectin it was of interest to study their action on other pectic substances such as sodium pectate and pectic acid.

(A) *Sodium pectate.* Preliminary experiments showed that the action of iodine on sodium pectate was very slow. Sodium pectate (2 g.) was dissolved in water and the solution divided into eight equal parts. Each part was treated with an excess (20 ml.) of $N/10$ iodine solution for varying periods at different temperatures, the excess of halogen was removed, and the sodium pectate was precipitated by alcohol (2 vols.), washed, dried at 100° for 20 hours and weighed (Table III).

Table III. *Action of iodine on sodium pectate.*

Time of treatment	Temperature (° C.)	Yield of sodium pectate recovered (g.)
Control	Room	0·24
24 hours	,,	0·24
72 ,,	,,	0·21
120 ,,	,,	0·20
240 ,,	,,	0·19
24 ,,	40–50*	0·22
72 ,,	40–50*	0·20
48 ,,	90*	0·20

* A fresh quantity of $N/10$ iodine solution was added when the solution became almost colourless.

Approximately 20 % of sodium pectate was destroyed in 10 days at ordinary temperatures, in 3 days at 40–50° and in 2 days at 90°.

(B) *Pectic acid.* 0·2 g. samples of pectic acid were weighed in a flask, suspended in water and treated with 20 ml. of $N/10$ bromine or iodine solution as shown in Table IV. (More halogen was added from time to time to replace the loss at higher temperatures.) Alcohol (2 vols.) was then added to precipitate any pectic acid dispersed in solution; the precipitate was collected, washed with alcohol, dried at 100° for 20 hours and weighed (Table IV).

Table IV. *Action of halogen on pectic acid.*

	Time of treatment	Temperature ° C.	Yield of pectic acid recovered g.
1. Control	—	—	0·20
2. Treatment with iodine	2 days	Room	0·20
3. ,,	3 ,,	,,	0·19
4. ,,	10 ,,	,,	0·19
5. ,,	3 ,,	50	0·18
6. ,,	6 ,,	50	0·17
7. ,,	3 ,,	90	0·16
8. Treatment with bromine	3 ,,	50	0·17
9. ,,	5 ,,	50	0·16

The action of halogen on pectic acid is negligible at room temperature and slight even at higher temperatures; this is probably due to its insolubility.

Action of halogens on the impurity isolated from a commercial Citrus *pectin.*

The "impurity," probably hemicellulose, from commercial pectin was obtained by allowing the pectin solution to stand overnight with sufficient lime water to render the solution just alkaline. After removing the precipitated calcium pectate, excess calcium was removed by adding oxalic acid till just

neutral, filtering and precipitating the filtrate with alcohol. 20 g. *Citrus* pectin gave 0·75 g. of the "impurity," *i.e.* 3·75 %; the latter contained about 23 % inorganic matter.

This isolated impurity (0·9 g.) was dissolved in water, the solution divided into three equal parts and then treated with excess of iodine or bromine solution at 40° for 6 hours. At the end of that period, alcohol (2 vols.) was added, and the insoluble impurity recovered was dried at 100° for 20 hours and weighed. As treatment with iodine failed to destroy about 33 % of the impurity the experiment was repeated, increasing the time of reaction to 12 hours. It was extraordinary to find that about 30 % of the impurity was still unattacked. This was possibly due to some change, physical or chemical, in part of the impurity during isolation. That this was actually the case was proved by an indirect procedure. A sample of pectin was first treated with iodine as on p. 456, and an attempt to isolate the impurity, by the method described above, was made. The product isolated from 20 g. of purified pectin weighed about 0·1 g. and consisted entirely of inorganic matter.

The results of treatment of the impurity with halogens are recorded below.

	Yield of ash-free impurity recovered g.
1. Control	0·17
2. Treatment with iodine for 6 hours	0·08
3. Treatment with iodine for 12 hours	0·07
4. Treatment with bromine for 6 hours	0·01

Application of the above methods to the preparation of pure pectin from plant materials.

The method was used in the preparation of very pure samples of pectin from *Citrus* fruits (grape fruit and lemon). The white inner rind of these was used, and extraction was carried out by means of hot *N*/75 HCl. (Extraction with water gave very small yields, and 0·5 % ammonium oxalate removed an excessive amount of non-pectic substance.) The extraction was carried out by adding about 10 times the quantity of acid to the powdered rind, and after keeping at ordinary temperature overnight, boiling for 1–2 hours on a water-bath; the extract was then filtered off at the pump. The cooled pectin extract was then directly treated with a fair excess of *N*/10 bromine water or *N*/10 iodine solution (iodine is not as satisfactory as bromine, for reasons already stated), and kept for 2 days at room temperature. The excess of halogen was then removed by ether, the pectin precipitated with alcohol and treated in the usual manner.

In certain cases, the method detailed above gave a slightly coloured product, owing to the presence of certain impurities which gave red to violet colorations with iodine. Also in other cases impurities which readily absorb halogens were present, which necessitated the use of a large quantity of the oxidising agent. Both the above difficulties could, however, be avoided if the pectin was precipitated by alcohol immediately after extraction, redissolved in water and then treated with the oxidising agent.

The results of extraction and purification of grape fruit and lemon pectin are recorded in Table V.

Determinations of total furfuraldehyde and the uronic anhydride content of the several samples of pectin, after purification by the methods evolved, were

Table V.

Sample of pectin	% yield (calculated on wt. of air-dried rind)	MeO % (Mean of two determinations)	Ca pectate % (Mean of two determinations)	Remarks
Series I:				
1. Lemon pectin without purification	25	9·4	95	Deep yellow-coloured product
2. Lemon pectin directly treated with iodine	21·8	11·4	100·2	Slightly coloured product
3. Same as 2 with bromine	20·8	11·5	100·4	White product
4. Lemon pectin precipitated after extraction, redissolved and then treated with iodine	20·0	11·7	100·6	White product
5. Same as 4 with bromine	18·9	11·6	100·7	White product
Series II:				
1. Grape fruit pectin without purification	24·1	9·8	95·3	—
2. Grape fruit pectin directly treated with iodine after extraction	20·7	11·7	100·6	—
3. Same as 2 with bromine	20·0	11·6	100·7	White product
4. Grape fruit pectin precipitated after extraction, redissolved and then treated with iodine	18·0	11·7	100·8	White product
5. Same as 4 using bromine	17·2	11·7	100·8	White product

carried out in order to test the agreement between the values obtained and those required by the formula given to the basal molecule of pectic acid by Nanji, D. R. *et al.* [1925] (Table VI).

Table VI.

	Furfuraldehyde %		Uronic acid anhydride %	
	Found	Calculated*	Found	Calculated*
Commercial pectin I	20·28	—	69·60	—
,, II	20·40	—	69·80	—
Grape fruit pectin	20·38	20·45	69·80	69·60
Lemon pectin	20·31	—	69·70	—

* Based on Nanji, Paton and Ling's formulae.

Discussion.

The purification of pectin with bromine and iodine in aqueous solution gives a rapid method of preparing pectin in a state of extreme purity, both from fairly pure commercial samples of pectin and also from *Citrus* fruits. The method would be particularly useful for investigations involving the structure of pectin, for unless a perfectly pure uniform sample of pectin were available conclusions regarding its ultimate constitution would have to be accepted with reserve. It is intended to extend the method to pectins from sources other than *Citrus* fruits.

The furfuraldehyde and carbon dioxide figures for purified pectin, as well as the action of halogens on sodium pectate and pectic acid, clearly show that no violent change has been produced in the pectins themselves by the action of halogens. The stability of the methoxyl groups in pectin towards oxidising agents is remarkable. Some evidence is already at hand to show that the fully methoxylated pectic acid is more stable to oxidation than the partly de-esterified pectin.

A full discussion of the function of methoxyl groups in pectin and the part they play in the genesis of pectin must be reserved till the work now in hand is completed; but it may be suggested that their function appears to be to prevent biological oxidation of pectin in plants.

SUMMARY.

1. Samples of pectin prepared from *Citrus* fruits and containing 90–95 % pectin can be further purified by treatment with bromine water or iodine at temperatures between 20° and 40°.

2. Chlorine water under similar conditions causes breakdown of the pectin: dilute hydrogen peroxide is without action.

3. The furfuraldehyde and carbon dioxide yields from pectins purified by this method agree with those usually accepted for highly purified pectin.

4. The methoxyl content of certain samples is found to approach very nearly that required by a fully methoxylated pectic acid.

5. The halogens have little action on sodium pectate and pectic acid.

The authors wish to record their thanks to the Coleer Pectin Company, New York and the California Fruit Growers Exchange for supplying commercial samples of *Citrus* pectin.

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LXV. THE SUPPLEMENTARY RELATIONSHIPS BETWEEN THE PROTEINS OF WHEAT AND RYE BREADS AND THOSE OF THE PEA (*PISUM SATIVUM*).

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It has been reported by Kon and Markuze [1931] that a supplementary relation exists between the proteins of soya bean flour and those of wheat and rye flour. The present paper deals with the effect of an addition of pea meal (from *Pisum sativum*) to the two latter substances on the biological value of their proteins.

Experimental.

The biological values of the proteins of pea meal, rye bread (baked from 70 % rye flour by the Municipal Bakeries in Warsaw), of two mixtures of wheat rolls and pea meal and of two mixtures of rye bread and pea meal have been determined by the method of Osborne *et al.* [1919].

The bread was dried at 35° and the dry product ground in a mill. The peas were boiled, dried and ground. The following mixtures were used: wheat-pea mixture I; wheat rolls 80 %, pea meal 20 %; wheat-pea mixture II; wheat rolls 60 %, pea meal 40 %; rye-pea mixture I; rye bread 80 %, pea meal 20 %; rye-pea mixture II; rye bread 60 %, pea meal 40 %. The nitrogen contents of the above products are given in Table I. The composition of the diets is given in Table II. The biological values of the proteins were, in most cases, estimated at

Table I.

Product examined	Protein content (calculated on dry wt.) N × 6·25 (%)	Ratio of bread- to pea-proteins
Rye bread from 70 % rye flour	7·4	—
Pea meal	26·2	—
Wheat-pea mixture I	14·0	1·8 : 1
Wheat-pea mixture II	16·8	1 : 1·5
Rye-pea mixture I	11·2	1·1 : 1
Rye-pea mixture II	14·6	1 : 2·4

several levels of protein intake. Albino rats bred in the laboratory were used in the experiments. The technique of the tests was as described by Kon and Markuze [1931]. The results will be found in Table III.

It will be seen from the tables that for the pea-meal-proteins alone a biological value of from 1·2 to 1·4 was found at 3 levels of protein intake. On the other hand the values found for the wheat-pea mixture I were from 1·6 to 1·9, while

Table II.

All values refer to dry weights

Source of protein	Composition of diets			
	Level of protein intake ($N \times 6{\cdot}25$)			
		7·1%		
Rye bread from 70% rye flour	Bread	95		
	Salts*	5		
	Level of protein intake ($N \times 6{\cdot}25$)			
		6·2%	8·2%	12·5%
Pea meal	Pea meal	22·9	30·5	45·8
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	62·1	54·5	39·2
	Level of protein intake ($N \times 6{\cdot}25$)			
		6·1%	8·8%	11·2%
Wheat-pea mixture I	Mixture	42·8	57·1	80·0
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	42·2	27·9	5·0
	Level of protein intake ($N \times 6{\cdot}25$)			
		6·3%	8·1%	11·0%
Wheat-pea mixture II	Mixture	35·7	47·6	65·5
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	49·3	37·4	19·5
	Level of protein intake ($N \times 6{\cdot}25$)			
		6·2%	8·2%	10·5%
Rye-pea mixture I	Mixture	53·5	71·4	95·0
	Butter fat	10·0	10·0	—
	Salts	5·0	5·0	5·0
	Potato starch	31·5	13·6	—
	Level of protein intake ($N \times 6{\cdot}25$)			
		6·3%	8·5%	11·8%
Rye-pea mixture II	Mixture	41·0	55·0	75·5
	Butter fat	10·0	10·0	10·0
	Salts	5·0	5·0	5·0
	Potato starch	44·0	30·0	9·5

* Steenbock and Nelson's [1923] salt mixture No. 40.

the second wheat-pea mixture gave at all three levels a value of at least 2·0, *i.e.* twice that found by Kon and Markuze [1931] for the proteins of wheat rolls. In all cases the biological value of the proteins of the mixtures exceeds that of the proteins of the separate constituents.

A similar supplementary relation was also found for the rye-pea mixtures. In the case of the rye-pea mixture I the biological value was 2·0 and 2·1 for the two lower levels and 1·6 for the highest level; it varied from 1·5 to 1·6 for all three levels of the second mixture. Rye bread alone gave a definitely lower value of 1·1. Owing to its low nitrogen content this bread could be fed only at one level of protein intake.

Results similar to those found in the case of wheat-pea mixtures have been observed when wheat gluten was fed to rats either as the sole source of protein or combined with pea meal. The mixture was composed of 80 % of pea meal and 20 % of wheat gluten (containing 84·2 % of protein). The two diets were fed

Table III.

Source of protein in diet	Protein ($N \times 6{\cdot}25$) content* %	Rat no.	Days	Total intake: Food* g.	Total intake: Protein* g. $= a$	Initial wt. g.	Gain in wt. g. $= b$	Biological value of protein b/a	Mean
Rye bread from 70% rye flour	7·1	827 ♀	31	236·8	16·8	66	14	0·83	1·14
		902 ♀	31	346·5	24·6	61	30	1·22	
		903 ♀	31	329·6	23·4	64	27	1·15	
		830 ♀	31	303·1	21·5	69	29	1·35	
Pea meal	6·2	786 ♀	31	183·1	11·3	46	14	1·24	1·21
		789 ♂	31	177·3	11·0	45	13	1·18	
	8·2	785 ♀	31	200·0	16·4	45	20	1·22	1·21
		788 ♂	31	223·3	18·3	49	22	1·20	
	12·5	784 ♀	31	242·0	30·2	40	41	1·36	1·38
		787 ♀	31	245·8	30·7	41	43	1·40	
Wheat-pea mixture I	6·1	745 ♀	31	302·5	18·4	55	35	1·90	1·92
		746 ♀	31	235·7	14·4	60	28	1·94	
	8·8	747 ♀	31	306·7	27·0	60	45	1·67	1·57
		749 ♂	31	285·8	25·1	58	37	1·47	
	11·2	748 ♀	31	349·8	39·2	63	66	1·68	1·77
		750 ♂	31	264·4	29·6	68	55	1·86	
Wheat-pea mixture II	6·3	752 ♀	31	285·9	18·0	54	39	2·17	2·11
		755 ♂	31	294·3	18·5	60	38	2·05	
	8·1	753 ♀	31	319·5	25·9	53	49	1·89	2·08
		754 ♀	30	330·8	26·8	53	61	2·27	
	11·0	740 ♀	31	330·8	36·4	59	80	2·20	2·00
		751 ♀	31	401·5	44·2	68	84	1·90	
Rye-pea mixture I	6·2	829 ♀	31	256·1	15·9	55	40	2·51	1·99
		833 ♂	31	250·3	15·5	75	25	1·61	
		881 ♀	31	304·8	18·9	49	35	1·85	
	8·2	831 ♀	31	311·2	25·5	62	55	2·16	2·07
		832 ♂	31	337·3	27·7	76	55	1·98	
	10·5	826 ♀	31	322·9	33·9	72	54	1·59	1·56
		837 ♂	31	299·2	31·4	56	48	1·53	
Rye-pea mixture II	6·3	840 ♀	31	288·5	18·2	56	30	1·65	1·56
		843 ♂	31	368·1	23·2	68	34	1·46	
	8·5	841 ♀	31	356·0	30·3	64	47	1·55	1·52
		844 ♂	31	355·0	30·2	71	45	1·49	
	11·8	842 ♀	31	341·8	40·3	74	56	1·39	1·47
		845 ♂	31	349·0	41·2	79	64	1·55	

* Calculated on dry weight.

Table IV.

Source of protein	Composition of diet* %		Protein ($N \times 6{\cdot}25$) content* %	Rat no.	Days	Total intake: Food* g.	Total intake: Protein* g. $= a$	Initial weight g.	Gain in weight g. $= b$	Biological value of protein b/a	Mean
Wheat gluten	Gluten	14·2	11·8	966 ♂	31	195·6	23·1	46	22	0·95	1·00
	Butter fat	10·0		967 ♂	31	187·2	22·1	43	20	0·90	
	Salts	5·0		968 ♂	31	244·0	29·0	47	33	1·14	
	Potato starch	70·8									
Gluten-pea mixture	Mixture	34·2	11·6	983 ♀	31	264·0	30·6	58	57	1·86	2·24
	Butter fat	10·0		984 ♀	31	294·2	34·1	57	78	2·29	
	Salts	5·0		985 ♂	31	285·0	33·1	56	85	2·57	
	Potato starch	50·8									

* Calculated on dry weight.

only at one level of protein intake; their composition and the results of the animal tests are given in Table IV. The biological value of the gluten proteins was found to be 1·0, which corresponds closely with the value obtained by Kon and Markuze [1931] for wheat roll proteins. The biological value of the proteins of the gluten-pea mixture is 2·2, *i.e.* about twice as great as in the case of wheat gluten alone and almost double that of the proteins of pea meal.

SUMMARY.

1. The biological values of the proteins of wheat gluten, of rye bread baked from 70 % rye flour, of pea meal and of mixtures in various proportions of pea meal with wheat rolls, rye bread and wheat gluten have been determined by the method of Osborne, Mendel and Ferry.

2. A supplementary relation exists between the proteins of wheat rolls and rye bread and those of pea meal.

3. The same is true in the case of a mixture of wheat gluten and pea meal.

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LXVI. THE ABSORPTION OF *n*-HEXADECANE FROM THE ALIMENTARY TRACT OF THE CAT.

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In continuance of previous studies of factors affecting the nature and amount of the unsaponifiable fractions of liver, it was shown that *n*-hexadecane is absorbed from the alimentary tract of the rat to the extent of 50 to 108 mg. daily, depending on the quantity of hydrocarbon included in the diet [Mahdi and Channon, 1933]. This result is of interest in relation to fat absorption, because it is opposed to current ideas that absorption of paraffinoid substances does not occur. A point of further importance arose in the course of this work. In one experiment, of 149 g. of hexadecane administered, only 116 g. were excreted, and of the 33 g. of hexadecane unaccounted for no more than an insignificant trace, if any, was present in the liver, whilst the unsaponifiable fraction of the carcass contained at most 1·85 g. of the hydrocarbon. The suggestion contained in this result of the ability of the rat to metabolise a normal hydrocarbon together with the surprising fact that the hydrocarbon failed to appear in the liver but did apparently find its way to the tissues, led us to investigate the matter further. Our first object was to confirm and extend these results by use of an animal which would provide more material for investigation, and also to study the distribution of the hydrocarbon in the tissues by its isolation in chemically pure form.

Experimental.

Hexadecane emulsified in a cream made by means of a machine for preparing a "synthetic" cream from butter and milk was administered to a cat. The amounts of hydrocarbon included, negligibly small at the start of the experiment, were gradually increased as the animal became accustomed to consuming the cream. The usual daily food provided—meat and fish—was withheld until the bulk of the cream had been consumed. At the end of each day, the vessel containing the cream residues was removed from the cage, and the contents were quantitatively transferred to a flask by means of ether. The combined residues were stored until the end of the experiment. By this means, 43·70 g. of hexadecane were presented to the cat during 29 days. A control animal received the same quantity of cream without added hexadecane.

Investigation of the tissues. At the end of the experiment and 24 hours after the last meal, the animals were killed by chloroform and skinned. The kidneys, heart, spleen, brain, liver, mesentery, omentum and perirenal fat were then removed. The muscle was then dissected from the skeleton. The tissues were heated with 5 % aqueous sodium hydroxide until dissolution occurred. The solutions were extracted quantitatively with ether. The fat obtained by

evaporation of the ethereal extract was saponified with alcoholic potassium hydroxide, and the alcoholic soap solution was poured into water and again extracted with ether. The crude unsaponifiable fractions were again saponified with sodium ethoxide to free them from possible contamination with fat, in view of the large amounts of some of the tissues used as starting material. The yields of unsaponifiable matter, together with its cholesterol content and iodine value, and the calculated iodine value of the non-sterol fraction of the unsaponifiable matter from the animal which received hexadecane are recorded in Table I.

Table I.

	Wt. of tissue g.	Unsap. matter g.	Unsap. matter %	Iodine value	Cholesterol as % of unsap. matter	Iodine value of non-sterol material (calculated)
Kidneys	56	0·2882	0·515	91·5	59·7	129·6
Heart	15	0·0827	0·551	42·4	20·0	36·5
Spleen	14	0·0919	0·656	57·1	58·9	44·6
Brain	26	0·6306	2·425	64·2	86·7	53·9
Liver	155	1·7943	1·158	66·5	58·9	67·4
Mesentery	56	0·4001	0·715	19·3	13·3	12·1
Omentum	176	1·0881	0·618	26·3	8·9	27·6
Perirenal fat	107	1·1313	1·057	28·0	13·3	22·2
Muscle	1531	3·2235	0·215	32·2	27·3	19·6
Skin	934	4·7691	0·511	23·5	26·1	8·6

These figures show that in the majority of these fractions the percentage of cholesterol in the unsaponifiable material is low, and this suggests the presence of excess of some unusual non-sterol constituent. That this is a saturated compound appears from the very low iodine values of the non-sterol fractions, particularly of the unsaponifiable materials of the mesentery, omentum, perirenal fat, muscle and skin which are 12·1, 27·6, 22·2, 19·6 and 8·6 respectively. The unsaponifiable fraction of the omentum was a deep yellow oil, those of the muscle and perirenal fat were almost liquid at room temperature, while the materials from the mesentery and skin were low-melting solids. In view of the small amounts of materials available, isolation of any hexadecane present could only be attempted in the case of the omentum, muscle, perirenal fat, skin and liver. These fractions were submitted to distillation *in vacuo* in Claisen flasks of suitable size. At the end of the distillations, the flasks were cooled and the residues quantitatively transferred by ether to small flasks, dried at 100° *in vacuo* and weighed. Because of its high cholesterol content (58·9 %), the liver material was dissolved in light petroleum and much of the cholesterol removed by crystallisation at 0° before distillation. The remainder of the cholesterol was quantitatively removed by means of digitonin. In Table II, the results of the

Table II. *Distillation of the unsaponifiable fractions.*

	Unsap. matter g.	Distillate g.	Yield as % of unsap. matter	Residue g.	Wt. of distillate referred to the whole of each unsap. fraction g.
Omentum	0·541	0·259	47·9	0·284	0·521
Muscle	2·49	1·54	61·8	0·900	1·99
Perirenal fat	0·681	0·329	48·3	0·360	0·547
Skin	4·21	1·27	30·2	2·91	1·44
Liver*	0·619	Trace	—	0·609	—
					4·5 g

* Sterol-free.

distillations are recorded, and the yields of distillate in the whole of each unsaponifiable fraction are shown in the last column.

The distillates were all obtained over a range of 152–166° at 18–21 mm. The hexadecane administered had B.P. 152°/12 mm., and bearing in mind the difficulties of distilling small amounts of materials such as these, the distillates could be considered as reasonably homogeneous. All the corresponding unsaponifiable fractions from the control cat were submitted to an identical distillation procedure, but in no case was any distillate obtained. Analysis of these fractions gave the following figures: Skin: C, 85·6; H, 14·47 %. Omentum: C, 84·23; H, 15·06 %. Muscle: C, 85·35; H, 14·52 %. Perirenal fat: C, 84·90; H, 14·98 %. $C_{16}H_{34}$ requires: C, 84·85; H, 15·15 %. These analytical figures indicated that all the fractions were fairly pure hexadecane. The fractions were therefore mixed, transferred quantitatively to a test-tube and treated twice with an equal bulk of concentrated sulphuric acid at 130° for 15 minutes with constant shaking to remove any contaminating oxygen compounds. The oil was then frozen out by cooling to 0°, transferred to a funnel with ether and washed, first with dilute alkali to remove sulphuric acid and sulphonic acids, and finally with water until the washings were neutral. After removal of the solvent the residue was dried at 100° *in vacuo*; 2·70 g. colourless oil were obtained having the following constants:

	M.P.	B.P.	Mol wt. (Rast)	C	H	Δ_4^{24}	Δ_4^{18}
Found	18	288	230, 234	84·73	14·97	0·772	—
Required for $C_{16}H_{34}$	18	288	226	84·85	15·15	—	0·775

These data show that the 2·70 g. of purified material was hexadecane. Unavoidable losses occurred in the treatment with sulphuric acid, but the isolation of the greater part of the material in chemically pure form and the carbon and hydrogen figures on the crude distillates leave little doubt that the latter were almost entirely hexadecane.

The amount of hexadecane absorbed.

Preparation of the unsaponifiable fraction from the cream residues yielded 12·06 g. of a pale yellow mobile liquid. Treatment with sulphuric acid by the method already described yielded 11·04 g.

The faeces, which had been collected throughout the experiment and stored under alcohol, were worked up for their unsaponifiable matter by the method used by Mahdi and Channon [1933]. The yield was 7·88 g. of a brown liquid which had an iodine value of 12·2 and contained 26·6 % of sterol precipitable by digitonin. The residue, which weighed after analysis 7·4213 g., was subjected to three successive treatments with sulphuric acid and yielded finally 3·6 g. of hexadecane. This was equivalent to 3·81 g. from the whole of the faecal excretion. Thus of 43·70 g. of hexadecane presented to the animal, 12·06 remained in the unconsumed food and 3·81 g. were excreted. The cat thus absorbed 27·83 g. of the hydrocarbon in 29 days, or approximately 1 g. per day.

Discussion.

These results confirm and extend those previously obtained on rats. The failure to isolate the hydrocarbon from the liver is of interest, and there seems little doubt that it was present, if at all, in traces only. The quantitative removal of the sterol before distillation of the liver-unsaponifiable fraction was carried out in order to facilitate the isolation of any hydrocarbon present, and yet the

0·619 g. of sterol-free material yielded on distillation only a trace of an oil, too small an amount for investigation. Further, 0·541 g. of the material from the omentum, which still contained its sterol, readily yielded 0·259 g. of hexadecane on distillation. This seems therefore proof of the absence of the hydrocarbon from the liver. With this result may be contrasted those previously obtained with squalene [Channon, 1926], and with a medicinal liquid paraffin [Channon and Collinson, 1929]. In the former experiments, squalene was isolated from the livers of rats, and in the latter, definite though indirect evidence of the presence of liquid paraffin in the livers of rats was obtained, and this was confirmed by the isolation of about 1 g. of the paraffin from the liver of a pig which had received the paraffin for a period of 54 days.

Hence the unsaturated hydrocarbon squalene, and a liquid paraffin which was shown to be a mixture of polycyclic hydrocarbons, both appear in the liver when administered in the diet, whereas the normal aliphatic hydrocarbon hexadecane does not. It is difficult to interpret the significance of this finding, especially as work on the fat-soluble vitamins and allied substances of recent years has shown that the liver appears to be the tissue in which these substances are predominantly stored. Equally difficult of interpretation is the presence of the hexadecane in the other tissues. Although sufficient material was not available for its isolation from all the tissues, inspection of Table I makes it appear not impossible that it may have been present in all of them. Be that as it may, hexadecane constituted from 30·2 to 61·8 % of the unsaponifiable fraction of the omentum, muscle, perirenal fat and skin, with an average of 47 %. Hence hexadecane does not appear in the liver, but does appear in the tissues. One point worthy of notice is that if the results are referred to yields of hexadecane per kg. of fresh tissue, they become: perirenal fat 3·08 g., omentum 1·47 g., skin 1·36 g. and muscle 1·00 g. per kg. The result with muscle is rendered uncertain, however, because the pad of fat ventral to the xiphisternum was included with the muscle tissue. Thus, so far as these results can be interpreted at present, they suggest provisionally that the hydrocarbon is distributed in the tissues in proportion to the amount of fat which the tissue contains.

We have no positive evidence as to whether squalene and liquid paraffin, which do appear in the liver on administration, also find their way into the fat depots, and we are giving attention to this problem. If the suggestion that the concentration of hexadecane in the tissues depends on their fat content is correct, it would be in keeping with its absence from the liver, which contains an amount of neutral fat of the order of 1 % only. The unsaponifiable fractions of depot fat are so small in amount that they have been little investigated, and these results suggest that they would repay further study.

Of the 28·14 g. of hexadecane absorbed, 4·50 g. only were isolated from the tissues, and hence 24·74 g. were metabolised in 29 days. As to the intermediate steps involved in this process we have no information at present, and we are investigating this problem further by another line of approach. No positive information is as yet available as to how the body finally metabolises any of the unsaponifiable substances. The general tendency for such substances to appear in the liver may suggest that that tissue plays a predominant part in their metabolism, as it does in the case of the carotene-vitamin A conversion. On the other hand, the latter may be a process peculiar to the liver which may act merely as a temporary place of storage for unsaponifiable substances generally. If the liver were concerned in the breakdown of these substances, it is conceivable that its ability to deal with a chemically inert material such as an aliphatic hydrocarbon is limited. The accumulation in the liver of the amounts

of hexadecane found in the tissues of the cat used in this experiment could obviously not be allowed to occur, if for no reason other than that of the solvent action of such quantities of the hydrocarbon on the lipoid constituents of the liver cells.

SUMMARY.

1. A cat absorbed 28·14 g. of *n*-hexadecane when 33·64 g. were administered to it in cream over a period of 29 days.

2. Consideration of the iodine values and cholesterol contents of the unsaponifiable fractions prepared from the different tissues suggested that hexadecane was generally present in them.

3. The hydrocarbon was isolated in chemically pure form from the omentum, perirenal fat, muscle and skin; it could not be isolated from the liver. Its distribution in the tissues appears to be related to their fat content.

4. The relationship of these findings to those previously obtained in experiments with squalene and with a liquid paraffin which was a mixture of polycyclic hydrocarbons is discussed.

5. The animal can metabolise *n*-hexadecane.

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LXVII. SOME FACTORS WHICH AFFECT THE ASSAY OF VITAMIN A BY THE SPECTROGRAPHIC METHOD.

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(*Received January 13th, 1934.*)

The physical methods of vitamin A assay have received much attention in the last few years but the trustworthiness of these determinations is still not clearly defined. Before discussing the present work, which deals with one aspect of the estimation of the vitamin, it may be of value to summarise briefly the present position.

The characteristic absorption spectrum of vitamin A is considered to be a broad continuous band with its head at 3280 Å. The intensity of the absorption at this wave-length in oils containing the vitamin has been found to agree very satisfactorily with the biological estimations [Coward *et al.*, 1931; 1932; Chevalier and Chabre, 1933]. This implies that constituents of the oil other than vitamin A absorb a proportional amount of light at 3280 Å.—or are transparent.

The blue colour obtained with the antimony trichloride reagent has been the subject of a large volume of work, and it now appears that the colour measured in the tintometer is the effect of two absorption bands, one at 5720 Å. and one at 6060 Å., whose positions are shifted by approximately 120 Å. towards the red in concentrated specimens. The intensities of these two bands have been compared with the intensity of absorption at 3280 Å. of the oil itself by Coward *et al.* [1931] and Gillam and Morton [1931], and it has been shown that while the intensities of the 5720 Å. band (in the antimony trichloride colour) and the ultra-violet band (in the oil itself) are always similar, the intensity of the 6060 Å. band does not appear to be so related. Furthermore, on oxidative treatment the latter band may be augmented in intensity leaving the other unaffected. As a result of their experiment, Heilbron *et al.* [1931] have suggested that only the band at 5720 Å. in the colour test is valid as a measurement of vitamin A activity.

The conditions which influence the intensities of these two bands of the colour are important in considering the validity of the blue test. The 6060 Å. band may be increased in intensity by keeping, by mild oxidative treatment with ozone, hydrogen peroxide and benzoyl peroxide and by saponification. It may be decreased in intensity by the addition of the antimony trichloride reagent in two stages, by the addition of pyrrole, indole, skatole or an unsaturated acid isolated from the saponifiable fraction of cod-liver oils [v. Eekelen *et al.*, 1933; Heilbron *et al.*, 1931]. The evidence points to the possibility of inhibition of the 6060 Å. band, but the improbability of inhibition of the 5720 Å. band. Assuming that the 5720 Å. band arises from vitamin A, it is necessary to adjust the conditions of conducting the "blue test" in order that the 6060 Å. band shall always bear the same relation to the 5720 Å. band.

For untreated cod-liver oils this appears to be the case, the ratio 6060 Å. band : 5720 Å. being 1·35 : 1, though considerable fluctuation about this mean value occurs (Gillam and Morton). If the absorption at 6060 Å. is caused to attain its maximum value by oxidative treatment a rather more constant ratio is attained (Heilbron *et al.*), but the mean value of the figures given the present writer finds to be 1·45 : 1 which differs from the previous ratio quoted by less than the estimated experimental error. This appears to the writer to indicate that in many cod-liver oils there can be very little inhibitor substance affecting the 6060 Å. band. In these oils saponification should not increase materially the absorption of the 6060 Å. band. In those cases where this band may be increased, we must infer that the ratio is less than about 1·40 : 1 and in order to attain the constant ratio, some means (*e.g.* saponification) must be used to enhance the 6060 Å. band. From these data it appears that in order that the colour test may measure variations in the 5720 Å. band, the determination must be made on the unsaponifiable matter in order that a constant ratio between the two bands of the colour test may be attained.

There is, however, another aspect of the problem. v. Eekelen *et al.* [1932; 1933], have stated that by an adsorption technique it has been possible to separate the two chromogens of cod-liver oils, which give rise to the two bands of the colour test. Only the fraction giving rise to the 6060 Å. band has been found to be active biologically. These experiments are at variance with the more circumstantial evidence which has been already summarised. The conclusions do not in any way affect the technique of making colour tests, however, since in each case the aim must be to cause the 6060 Å. band to attain its maximum value.

Measurements of vitamin A content on the basis of absorption at 3280 Å. involve, however, a purely empirical assumption that irrelevant absorption at this wave-length bears a constant relation to the relevant absorption for all oils or is negligible in comparison with it. With a view to attempting to estimate this irrelevant absorption, cod-liver oils have been aerated at 100° and the absorption determined at intervals. At the same time a sample of the oil was saponified and the absorption of the unsaponifiable material determined. In Fig. 1 a typical curve is shown for an oil of blue value 22 (B.P. test) referred to 20 % solution. Curve I which refers to the absorption at 3280 Å. of the oil on aeration may be regarded as compounded of two curves. By producing back the straight portion of the curve to the axis representing absorption and subtracting the two curves, curve III is obtained. From curve II which represents the absorption of the unsaponifiable matter after the oil has been aerated for varying times (referred to 1 % of oil) it is seen that the absorption attained a constant value after 5 hours, by which time no blue colour was obtained with antimony trichloride. This constant value may be taken as representing the absorption of unsaponifiable matter which is not vitamin A, and by subtracting it from curve II the true values for vitamin A should be obtained. This last curve is almost

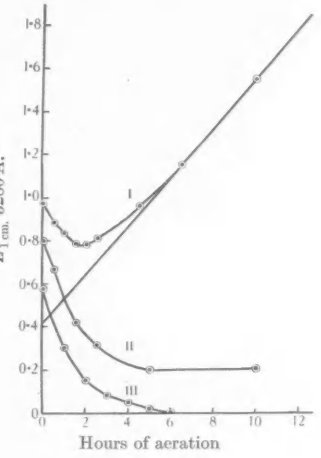

Fig. 1.

identical with curve III which is logarithmic. It is, therefore, assumed that the extrapolated straight line in curve I represents the manner in which the absorption at 3280 Å. of the non-relevant part of the oil varies with time of aeration. If this is true, the intercept of this line on the axis representing $E^{1\%}_{1\,\mathrm{cm.}}$ provides a value for the absorption of the constituents other than vitamin A in the original oil, and in this case it amounts to approximately 40 % of the total absorption. The curves shown draw attention to the possibility of serious inaccuracies arising from the measurement of absorption at 3280 Å. as the sole criterion of vitamin A potency. Furthermore, the effect of previous ageing of the oil by oxidation probably has the effect of increasing the irrelevant, as well as decreasing the relevant, absorption at 3280 Å. The actual variation in absorption by oxidative change at room temperatures cannot of course be gauged by these curves since the temperature coefficients of the two reactions involved will probably be different. From the published figures of Heilbron *et al.* [1931] it appears that in some cases at least an actual increase of total absorption may occur after storing.

In Fig. 2 two further curves of aeration are given, curve I for an unusually potent cod-liver oil of blue value 112, and curve II for a weak oil of blue value 14. In each case a similar curve to that in Fig. 1 has been obtained, the linear

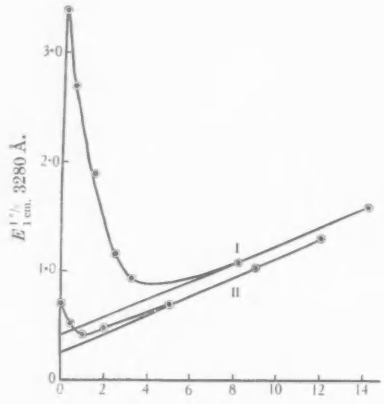

Fig. 2.

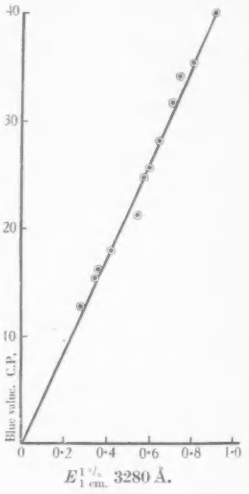

Fig. 3.

portions of which are approximately parallel and of similar initial values. Again, in the less potent oil the irrelevant absorption is of the order of 40 %, while in the potent oil it is approximately 12 %.

In a recent paper Dann and Moore [1933] have shown that the time of saponification is a factor which affects the absorption of the fatty acids from cod-liver oils. Edisbury *et al.* [1933] have suggested "cyclisation" as the cause. While a discussion of the chemical nature of the changes brought about by aeration treatment lies outside the scope of this paper, it is suggested that the increase in absorption at 3280 Å. by aeration may arise from a similar cause since the absorption at lower wave-lengths is increased very considerably, but by the

technique used it would not have been possible to detect the fine structure bands noted by these authors.

It would seem that the absorption at 3280 Å. can only be regarded as measuring the vitamin A activity for oils more potent than that shown in Fig. 2, curve I. For less potent oils the "non-vitamin A" absorption at 3280 Å. constitutes a considerable proportion of the total absorption. The very real parallelism, however, between the absorption in this region and the biological tests for normal cod-liver oils cannot be dismissed as fortuitous. It has been found that the oils which have been so far examined by the aeration technique contain approximately 40 % of irrelevant matter, as measured by the intercept, provided that the potency is of the order of 10–20 blue units. In these circumstances the measured absorption of the oil will be proportional to the amount of vitamin A present.

In Fig. 3, a curve for the blue values and absorption values of the unsaponifiable fraction of cod-liver oils has been drawn. Of all the oils which have been examined none has deviated from this curve by more than 12 %. Josephy [1933] has recorded some results of a similar nature.

While it is not practicable to adopt the aeration technique here described, it appears to the author desirable to determine the absorption upon the unsaponifiable matter from the oil rather than upon the oil itself. In this way errors arising from previous oxidation of the oil are eliminated and there only remains the error due to absorption at 3280 Å. of irrelevant unsaponifiable matter.

SUMMARY.

The effect of oxidative change on the absorption at 3280 Å. of liver oils is discussed in so far as it affects the assay of vitamin A by the spectrographic method. The need for saponification of the oil in order to avoid errors arising from previous oxidation is emphasised.

The author is indebted to Prof. Drummond for his interest in the work and his helpful suggestions.

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LXVIII. THE ENZYMIC HYDROLYSIS OF PHOSPHATIDES.

III. THE HYDROLYSIS OF NATURAL AND SYNTHETIC PHOSPHATIDES.

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(*Received January 30th, 1934.*)

It has previously been shown [King, 1931] that an enzyme is contained in the kidney and intestinal mucosa which is capable of hydrolysing lecithin to liberate inorganic phosphate at an optimum p_H of 7·5. The enzyme appears to be present in other tissues to only a slight extent. Bone, which is very rich in an enzyme hydrolysing primary esters of phosphoric acid, is relatively feeble in its action against lecithin. Lecithinase appears to act most rapidly at body temperature (Fig. 1). It is fairly stable at a neutral reaction but is rapidly destroyed in an alkaline or acid medium. The concentration of substrate (Fig. 2) appears to

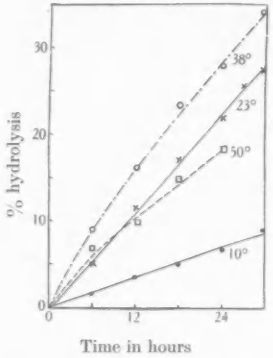

Fig. 1. Effect of temperature on the hydrolysis of lecithin:

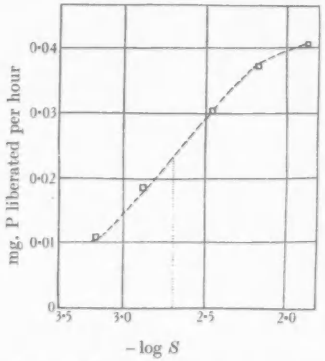

Fig. 2. Effect of substrate concentration on lecithinase hydrolysis:

10 ml. of lecithin emulsion at p_H 7·5 + 2 ml. of dialysed extract of intestinal mucosa.

affect the hydrolysis in a manner similar to that indicated by Michaelis and Menten [1913]. Owing to the slowness with which lecithin is broken down by the enzyme, it has been impossible to obtain any accurate measure of the initial velocity of hydrolysis. The results appear to indicate, however, that the substrate concentration giving half the maximum velocity corresponds to about 0·002 M ($-\log S$ 2·7), which is nearly the same as that found by Martland and

Robison [1927] for phosphatase, *i.e.* $<0{\cdot}003\,M$ [cited by Haldane, 1930]. It is the purpose of the present paper to record the results of a study of the action of this enzyme on other phosphatides, both natural and synthetic.

Lecithin, kephalin and phosphatidic acid.

Liver-lecithin behaved much like egg-lecithin, while kephalin showed a lower rate of hydrolysis with not so marked a dependence on hydrogen ion concentration. A sample of phosphatidic acid from cabbage leaf [Chibnall and Channon, 1927] was kindly supplied to us by Prof. A. C. Chibnall. The formula of phosphatidic acid is represented as corresponding with that of lecithin with the choline absent. It showed a fairly high rate of hydrolysis, but the relation of the amount of hydrolysis to the p_H was not definite as in the case of lecithin (see Table I).

Table I. *Hydrolysis of natural phosphatides by extract of the small intestine.*

(mg. inorganic phosphorus.)

5 ml. borax-boric acid or borax-succinic acid buffer, 5 ml. 0·25 % emulsion of phosphatide and 2 ml. enzyme solution. The hydrolysis was carried out in the presence of chloroform and toluene for 12 hours at 37·5° and was stopped by the addition of 2 ml. 25 % trichloroacetic acid. The inorganic phosphate in the filtrates was estimated by the colorimetric method.

	p_H	Control (1); buffer + phosphatide	Control (2); buffer + enzyme	Test; buffer + phosphatide + enzyme	Phosphate hydrolysed
Egg-lecithin	5·5	0·000	0·041	0·058	0·017
	6·5	0·000	0·037	0·082	0·045
	7·1	0·000	0·038	0·109	0·071
	7·5	0·000	0·039	0·119	0·080
	8·1	0·000	0·039	0·112	0·073
	8·9	0·000	0·038	0·091	0·053
	9·2	0·000	0·037	0·068	0·031
Liver-lecithin	5·6	0·000	0·040	0·056	0·016
	6·5	0·000	0·037	0·067	0·030
	7 1	0·000	0·038	0·091	0·053
	7·6	0·000	0·039	0·096	0·057
	8·0	0·000	0·039	0·095	0·056
	8·9	0·000	0·038	0·066	0·028
	9·2	0·000	0·037	0·056	0·019
Kephalin	4·3	0·020	0·035	0·069	0·014
	4·8	0·020	0·034	0·070	0·016
	5·0	0·021	0·034	0·083	0·028
	5·5	0·020	0·041	0·103	0·042
	6·5	0·021	0·037	0·103	0·045
	7·1	0·020	0·038	0·101	0·043
	7·6	0·020	0·039	0·101	0·042
	8·3	0·019	0·039	0·107	0·049
	8·9	0·020	0·038	0·090	0·032
	9·2	0·021	0·036	0·089	0·032
Phosphatidic acid	5·6	0·068	0·040	0·191	0·083
	6·6	0·069	0·037	0·193	0·087
	7·0	0·067	0·038	0·187	0·082
	7·5	0·067	0·039	0·185	0·079
	8·0	0·070	0·038	0·185	0·077
	8·9	0·073	0·038	0·185	0·074
	9·2	0·069	0·037	0·180	0·074

Lysolecithin.

Lysolecithin was prepared by the action of the venom of the fer de lance on the lecithin contained in egg-yolk [King and Dolan, 1933]. The material so prepared has marked haemolytic properties. The behaviour of lysolecithin with

both the intestinal and bone enzymes was investigated. The amounts of hydrolysis at different hydrogen ion concentrations and at different times, when lysolecithin was subjected to the action of an aqueous extract of rabbit intestinal mucosa, indicated that the maximum hydrolysis occurred at about p_H 7·8, which is a little higher than the corresponding value for lecithin, and that this relatively simpler phosphatide was hydrolysed about twice as fast as the parent lecithin.

Bromolecithin.

Bromolecithin was prepared by passing bromine vapour in a stream of carbon dioxide into a mixed ether and light petroleum solution of egg-lecithin and liver-lecithin [Levene and Rolf, 1926]. The precipitated material was purified by solution in ether and reprecipitation by light petroleum.

Analysis for phosphorus was made by the perchloric acid colorimetric procedure [King, 1932], for nitrogen by micro-Kjeldahl, and for bromine by the potash fusion method of Fendler and Stüber [1914], the silver bromide being collected by means of a porcelain filter stick. (Found: P, 2·02; N, 1·04; Br, 41·3 %. Calculated: for octabromoarachidyl-stearyl-lecithin: P, 2·12; N, 0·96; Br, 43·7 %; for octabromoarachidyl-palmityl-lecithin: P, 2·16; N, 0·97; Br, 44·6 %; for hexabromostearyl-stearyl-lecithin: P, 2·42; N, 1·09; Br, 37·5 %.)

The behaviour of bromolecithin in the presence of phosphate-liberating enzymes was in marked contrast to that of the lecithin from which it had been made. With a variety of enzyme preparations from several sources, bromolecithin was practically completely hydrolysed in 24 hours, while lecithin was attacked only to the extent of 20 % in the most favourable instance. This peculiar property of greatly increased susceptibility to enzymic hydrolysis resulting from bromination of lecithin is probably related to an increased affinity of the enzyme for the phosphatide, due to the change in the fatty acid side-chain. The effect of p_H on the enzymic hydrolysis of bromolecithin is shown in Fig. 3.

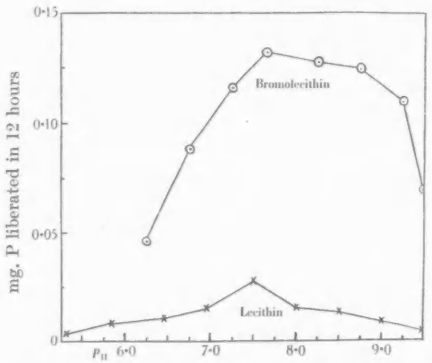

Fig. 3. Effect of p_H on hydrolysis of bromolecithin by cat-kidney extract. Each test contained 0·16 mg. of phosphatide-P, and 0·5 ml. of enzyme. Veronal-acetate buffer; hydrolysis at 38° for 12 hours.

The rates of hydrolysis of bromolecithin, lysolecithin and the parent lecithin are given in Table II, the hydrolysis constant being calculated from $k = \frac{1}{t} \log \frac{a}{a-x}$. The velocity of hydrolysis for bromolecithin was about 10 times as great as for

lecithin in the case of the intestinal enzyme, and 100 times as great with bone-phosphatase. The bone-enzyme, moreover, gave with bromolecithin about twice the velocity of hydrolysis that was obtained with the intestinal enzyme. This is in marked contrast to the action of the two enzyme preparations on lecithin. The velocities for bromolecithin were in about the same ratio as the phosphatase activities of the two preparations measured against glycerophosphate.

Table II. *Comparative rates of enzymic hydrolysis of phosphatides.*

	Lecithin		Lysolecithin		Bromolecithin	
Hours	% hydrolysis	k	% hydrolysis	k	% hydrolysis	k
	(1) Rabbit intestinal extract.					
½	1·0	0·0072	1·6	0·0138	9·1	0·0834
1	1·2	0·0054	2·0	0·0090	17·9	0·0860
2	2·4	0·0054	2·4	0·0054	29·0	0·0744
4	3·2	0·0036	4·8	0·0054	44·8	0·0645
7	4·8	0·0031	7·9	0·0052	59·9	0·0567
	Average	**0·0049**		**0·0078**		**0·0750**
	(2) Rat bone extract (phosphatase).					
½	—	—	0·7	0·0070	15·1	0·1424
1	0·4	0·0018	0·8	0·0036	28·6	0·1461
2	0·8	0·0018	1·1	0·0025	50·0	0·1505
5	1·2	0·0010	1·3	0·0012	65·9	0·0934
7	1·5	0·0009	1·6	0·0010	70·2	0·0751
	Average	**0·0014**		**0·0031**		**0·1215**

Synthetic phosphatides.

Through the kindness of Dr Adolf Grün, of the Hoffman-La Roche Company, Grenzach, Germany, we were able to compare samples of the synthetic phosphatides described by Grün and Limpächer [1927]. Synthetic distearyllecithin did not show the same general effect on enzymic hydrolysis as did the natural lecithin (Table III). The rate of hydrolysis was not so great and the p_H effect

Table III. *Influence of* p_H *on the enzymic hydrolysis of natural and synthetic phosphatides.*

(1) Natural egg-lecithin		(2) Synthetic lecithin (Grün)		(3) Distearyl phosphate (Grün)	
p_H	mg. P liberated	p_H	mg. P liberated	p_H	mg. P liberated
3·6	0·145	5·7	0·121	4·5	0·183
4·3	0·158	6·2	0·115	5·9	0·193
5·9	0·186	6·9	0·119	6·6	0·176
6·9	0·221	7·3	0·121	6·9	0·178
7·1	0·220	7·5	0·118	7·4	0·174
7·3	0·222	8·0	0·111	7·8	0·176
7·5	0·205	8·8	0·127	8·2	0·170
7·8	0·192			9·5	0·145
8·1	0·170				

was absent. It was thought that this difference might be due to the unsaturation and greater solubility of the natural product. This explanation was found to be untenable, however, by the discovery that hydrogenated lecithin has almost the same rate of enzymic hydrolysis as has the lecithin from which it is made (Table IV).

Synthetic distearyl phosphate showed very little relation between the p_H and the amount of hydrolysis. Since it is a primary ester of phosphoric acid,

Table IV. *Enzymic hydrolysis of hydrolecithin and lecithin.*
(mg. P liberated.)

Tests consisted of 2 ml. of enzyme (rat-intestinal extract), 5 ml. of veronal buffer and 5 ml. of emulsified phosphatide containing 0·086 mg. of hydrolecithin-P per ml. and 0·088 mg. of lecithin-P per ml. The hydrolysis took place at 38° for 48 hours.

p_H	Hydrolecithin*	Lecithin	p_H	Hydrolecithin*	Lecithin
5·8	0·114	0·114	8·0	0·203	0·194
6·5	0·151	0·158	8·5	0·187	0·188
7·1	0·208	0·206	8·9	0·161	0·170
7·5	0·201	0·202	9·4	0·052	0·082

* This sample of hydrolecithin was kindly given to us by Dr P. A. Levene. Other samples prepared in this laboratory by hydrogenation of egg- and liver-lecithins in the presence of palladium gave very similar results.

it might have been expected to behave like glycerophosphoric acid or acetonyl-glycerophosphoric acid, which are hydrolysed at an optimum p_H of 8·9, whereas lecithin, a secondary ester of phosphoric acid, is hydrolysed at an optimum p_H of 7·4 (diphenyl phosphate and diethyl phosphate are hydrolysed at a slightly lower p_H optimum). Comparative figures for the hydrolysis of natural and synthetic lecithin and distearylglycerophosphoric acid were obtained by incubation of 5 ml. of 0·25 % emulsion of the phosphatide mixed with an equal volume of buffer at several p_H values and 2 ml. of the enzyme solution for 48 hours. The amount of phosphate liberated from the egg-lecithin was considerably greater than that from the distearyl phosphoric acid and at the optimum p_H was almost double that liberated from the synthetic lecithin.

Discussion.

The extraordinary change induced in lecithin by bromination is not easily explained. It is a more common experience to find that a large increase in molecular weight (that of bromolecithin is almost double that of lecithin) leads to a decrease rather than an increase in reactivity. A possible explanation of this anomalous result of greatly increased susceptibility to enzymic hydrolysis may lie in a greater negativity of the alcohol part of the ester due to introduction of several bromine atoms into the side-chain. Among the simple primary esters of phosphoric acid those containing the more negative alcohol groups are most readily attacked by phosphatase; thus phenyl phosphate is much more rapidly hydrolysed than tolyl phosphate, and tolyl phosphate very much more rapidly than ethyl phosphate.

In the case of phosphatidic acid, the p_H effect was almost absent, and since phosphatidic acid is a primary phosphoric ester, it—as also distearyl phosphate—might have been expected to be hydrolysed optimally at p_H 8·9. Since, however, the phosphate-liberating enzyme is relatively unstable above p_H 8, while it is quite stable in more neutral solutions, the discrepancy may be explicable on the basis of destruction of the enzyme during the course of hydrolysis. If this be so, the length of the fatty acid chains may be the determining factor, since it probably conditions the solubility of the phosphatide and its consequent availability for enzymic hydrolysis. The shorter the carbon chains of the substituent groups, the more soluble the substance should be and the more it should behave like a simple primary phosphoric ester with a p_H optimum of 8·9. It is also possible that the nature of the fatty acid radicals may affect, through some mechanism akin to steric hindrance, the union of substrate and enzyme.

Thus the presence of the fatty acids in phosphatidic acid may hinder its union with the enzyme, giving a lower rate than would be obtained with an unsubstituted glycerophosphate, or with a glycerophosphate substituted with less complex groups than stearic and oleic. This, combined with destruction of the enzyme, might account for the failure of this product to be hydrolysed at the optimum p_H of 8·9.

If the difference between the behaviours of natural and synthetic phosphatides on enzymic hydrolysis were due to the unsaturation of the natural product, then phosphatidic acid, which is more unsaturated than egg-lecithin, might have been expected to show a higher rate of hydrolysis. Also, hydrolecithin, which ought to be very similar in chemical behaviour to the synthetic product if existing theory as to the structure of these substances be correct, might have been expected to behave like synthetic lecithin, instead of which it showed an enzymic hydrolysis almost identical with that of its parent lecithin. These facts would appear to cast some doubt on the similarity of the chemical configuration of the synthetic lecithin of Grün and Limpächer with the naturally occurring phosphatide; and further, if the chemical formula assigned by Grün and Limpächer to the synthetic product be correct, then the formula commonly used to represent the chemical structure of lecithin may be in error. The asymmetric formula which appears in most of the textbooks and represents lecithin as always being a derivative of α-glycerophosphoric acid, has been shown to be incorrect [Rae, 1934], and it is possible that a more drastic revision of the manner of representing the formula for lecithin may yet be necessary.

Summary.

An enzyme is contained in kidney and intestinal mucosa which hydrolyses lecithin from several sources with the liberation of phosphoric acid. Hydrolecithin is attacked as fast as its parent lecithin, kephalin and phosphatidic acid somewhat more slowly, the latter without showing any marked dependence on p_H. Synthetic lecithin and distearyl phosphate are attacked by the enzyme at a lower rate and without showing any p_H optima. Lysolecithin is hydrolysed about twice as fast as lecithin. Brominated lecithin is hydrolysed at a very much greater rate than is lecithin.

The thanks of the author are due to Miss M. E. Dolan and Miss M. M. Shaw for valuable assistance during the course of these experiments.

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LXIX. THE ULTRA-VIOLET SPECTRUM OF OXYHAEMOGLOBIN.

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(*Received February 5th, 1934.*)

A prominent band in the near ultra-violet spectrum of oxyhaemoglobin was discovered by Soret [1883] who referred to it as the "great" ultra-violet band. Gamgee [1895] pointed out that this band was also found in haemoglobin, carbon monoxide haemoglobin, nitrous oxide haemoglobin, haemochromogen, haematin hydrochloride, methaemoglobin and haematoporphyrin, but was absent from the spectra of bilirubin, hydrobilirubin and urobilin. The position of the peak of this oxyhaemoglobin band in the wave-length scale was found by Rost *et al.* [1909] to be 4100 Å. Newcomer [1919] placed it at 4140 Å. and Schumm [1921] at 4135–4140 Å.

In the case of carbon monoxide haemoglobin the absorption is greater, and the band is shifted towards the red, while the spectrum of acid haematin shows a similar band with the absorption not so pronounced and extending into the ultra-violet, obliterating a small peak at 3650 Å. which is characteristic of the oxy-haemoglobin spectrum.

Oxyhaemoglobin also has two bands with peaks at 3400 Å. and 2700 Å., and beyond 2400 Å. the absorption becomes general. Warburg and Negelein [1929] on measuring the absorption of light by the extent of chemical change which the absorbed energy produced, fixed the peak of the "great" band at 4300 Å. and found another prominent band at 2700 Å. in the case of oxyhaemoglobin, the latter band being lacking in all pure haem compounds.

Newcomer [1919] concluded that the similarity between the spectra indicated the presence of a common radical, but Hicks and Holden [1929] could not find any relationship between the valency of the iron in the pigments and their ultra-violet spectra. Anson and Mirsky [1930] state that pigments of similar chemical structure produce spectra of the same general form and of the same dimensions.

Macallum and Bradley [1930] pointed out that the absorption band occurring at 4100 Å. for crystalline oxyhaemoglobin of the horse does not appear in the spectrum of washed corpuscle suspensions containing the pigment in its normally occurring form. These observations have been verified and extended to the blood pigments of other species.

The instrument employed was a Bellingham and Stanley quartz spectrograph. The ultra-violet spectrum from 2100 to 4500 Å. was extended over 18 cm. upon the negative. The absorption measurements were determined by the use of a photoelectric recording photometer developed in this laboratory by Macallum and Coates [1933].

The blood was drawn from the animal into sodium citrate. In the case of the corpuscle suspensions the blood was diluted with mammalian saline so that the pigment concentration was adjusted to a predetermined value (0·2 % haemoglobin), these values being determined by Newcomer's method. In the case of the haemoglobin, the corpuscle suspension was laked with ether, the haemoglobin values being adjusted as stated above.

Typical results may be illustrated by the data obtained from rabbits' blood (Fig. 1).

1. Unlaked blood. The absorption curve shows a slight elevation about 4500 Å. then a fall towards 4300 Å.; between 4300 and 4000 Å. there is another slight rise terminating about 4000 Å. There is another slight rise at 3900 Å. The curve then becomes level to 3500 Å.

2. Laked blood. The absorption curve is fairly level to 4200 Å. where it rises sharply to a peak about 4100 Å. and then drops off gradually to the original level at 3900 Å. The curve is then level to 3700 Å. where it recedes slightly and then rises at 3600 Å. and comes back to the original level at 3500 Å. The laked blood at the height of the great ultra-violet band (4100 Å.) shows about 40 % absorption for the solution used.

In comparing the laked and unlaked oxyhaemoglobin, it is seen that the laked shows greater absorption at any given wave-length.

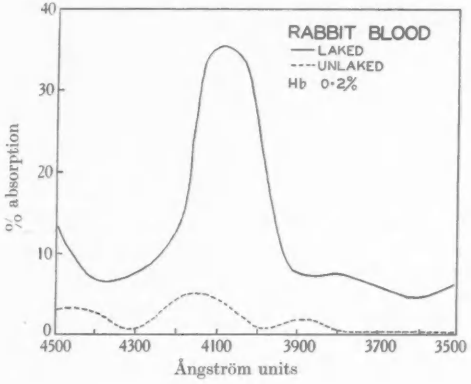

Fig. 1.

Oxyhaemoglobin from other species. For the sake of convenience the beginning of the great ultra-violet band has been designated A, the peak B and the end C, the figures representing Å.

1. Laked blood.

	A	B	C
Rabbit	4200	4100	3900
Goose	4300	4100	3910
Human	4300	4100	3850
Turtle	4300	4100	3900
Horse	4200	4100	3800

2. Unlaked blood. The absorption spectra do not show any marked band.

The spectrum of rabbit-oxyhaemoglobin, in the visible region, was examined

both in the corpuscle suspension and in the laked blood and the position of the α and β bands determined. The position of the bands was fixed as follows:

	α band (Å.)	β band (Å.)
Laked blood	5780	5400
Unlaked blood	5780	5400

This experiment was repeated several times on rabbits' blood and indicates that there is no difference in the spectral bands of oxyhaemoglobin inside or outside the corpuscle.

Discussion.

Our observations have been made on oxyhaemoglobin solutions, and they have served to confirm the observations previously recorded. The great ultra-violet absorption curve in the oxyhaemoglobin solutions shows in most cases an origin about 4300 Å., a rise to a peak about 4100 Å. and then a drop back to 3900 Å. The unlaked blood does not show this great ultra-violet band in the region of 4100 Å. The rest of the curve remains much the same except that there is a slight rise in some cases at 4100 Å.; in all cases where this latter absorption occurred there was slight haemolysis, and it is believed that this absorption is due to traces of oxyhaemoglobin outside the corpuscle.

There is the possibility that the absence of the great ultra-violet band from the corpuscle suspensions might be due to the masking effects of the blood-serum, the corpuscle wall, material mixed with haemoglobin in the cell or material combined with haemoglobin in the cell but removed by ether-laking. As to the serum it has been shown by Lewis [1917] that the ultra-violet absorption spectrum of serum has two peaks at 2800 Å. and 2400 Å.; it represents several superimposed curves and does not resemble that of a pure compound, being due chiefly to protein. Furthermore, we have found that the great ultra-violet band of oxy-haemoglobin exists in laked whole blood as well as in aqueous solutions of crystalline oxyhaemoglobin.

The question as to whether the corpuscle wall itself may be a factor can be eliminated by the fact that blood laked by freezing and containing the corpuscle "ghosts" still displays the band in the ultra-violet. Removal of the "ghosts" by centrifuging does not affect the absorption band.

Cell stromata evidently play no part since the laked blood containing corpuscle contents displays the spectrum of pure oxyhaemoglobin.

Finally, since our early experiments were carried out on ether-laked samples it might be possible that this treatment had removed a lipoid fraction which was combined with the oxyhaemoglobin in the corpuscle and that lack of this portion might be responsible for the absence of the band under question. However, the fact that laking brought about by the physical processes of freezing and thawing, which do not remove any constituent of the cell, still gives the same spectrum as the ether-laked blood indicates that the presence of the band is independent of the absence of any ether-soluble substance.

Our results indicate that there is an essential difference between oxyhaemo-globin inside and outside the corpuscle. If the presence of absorption bands is to be interpreted as indicating a condition of desaturation then it must be assumed that oxyhaemoglobin outside the corpuscle is in a state of unsaturation while in its naturally occurring state in the cell it enters into combination with some of the cell constituents. The combination must be rather labile since it is disrupted by any force which alters the osmotic balance of the cell.

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LXX. THE OXIDATION OF PALMITIC ACID, BY MEANS OF HYDROGEN DIOXIDE IN THE PRESENCE OF A CUPRIC SALT.

PRELIMINARY COMMUNICATION.

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Palmitic is probably the most commonly occurring saturated fatty acid and is one of the fatty acid constituents of lipins; yet, although in the body it must undergo oxidation with extraordinary ease, it is very resistant to the action of the usual laboratory chemical oxidising agents. Even after being heated on a water-bath for 18 hours with a concentrated alkaline solution of potassium permanganate about 40 % of the original palmitic acid was recovered. A dihydroxypalmitic acid and oxalic, succinic and adipic acids were isolated from the reaction product together with acetic, butyric and possibly capronic and caprylic acids [Gröger, 1887]. Information does not appear to be available with regard to palmitic acid but oxidation of myristic acid with strong nitric acid resulted in the formation of a mixture of dibasic acids, amongst which oxalic, succinic, adipic and suberic were identified [Nördlinger, 1886].

A small proportion of palmitic acid was shown by Dakin [1908, 1] to undergo β-ketonic oxidation when its ammonium salt was distilled with hydrogen dioxide on a boiling water-bath. About 10 % of the original acid was thus converted into carbonic acid and methyltridecylketone; the addition of a considerable excess of the dioxide produced very little further effect. Clutterbuck and Raper [1925] showed that the γ- and δ-keto-acids were formed to about the same extent as the β-acid. On further oxidation of the γ- and δ-keto-acids, succinic acid was identified in the product [Clutterbuck, 1927].

Whilst very definite evidence can be obtained that the path of oxidation of the α-amino-acids *in vivo* lies through the α-keto-acids there is no evidence that β-keto-acids are formed as the intermediate products of oxidation of the higher fatty acids. Acetoacetic and β-hydroxybutyric acids accumulate when the mechanism for the oxidation of the fatty acids is overburdened or impaired, and the available evidence points to the β-hydroxy-acid being formed by reduction of acetoacetic acid in the tissues; but these two acids are mutually convertible in the body, and the possibility of hydroxylation being the process involved cannot be excluded.

There is no evidence of the existence in the body of any ketones such as those isolated by Dakin in his experiments on the oxidation of the fatty acids *in vitro*, and if the higher β-keto-acids are so readily oxidised it is difficult to understand why a mechanism which splits the original β-keto-acid so completely into acetic acid and an acid containing two less carbon atoms than the original should cease completely to function when the stage of acetoacetic acid is reached.

The process used in the body is so perfect that no intermediate substances can be identified.

Smedley-MacLean and Pearce [1931] showed that oleic and *n*-octanoic acids were rapidly and almost completely oxidised by the action of hydrogen dioxide in the presence of a cupric salt. In the present investigation the addition of a few drops of cupric sulphate solution to one of sodium palmitate has been found to produce an equally potent effect on the oxidation of palmitic acid by hydrogen dioxide. Contrary to our expectation, palmitic was as readily oxidised as oleic acid. Indeed the behaviours of oleic and palmitic acids when heated for 3 to 4 hours at 90° with a large excess of hydrogen dioxide and a few drops of the cupric solution were very similar. About 70 % of each acid was converted into carbonic and volatile fatty acids. In the earlier stages of the oxidation of palmitic acid volatile lactones giving propionic acid on treatment with strong sulphuric acid were present. Under milder conditions of oxidation, succinic acid was isolated from both oleic and palmitic acids in about the same amounts, and in the case of oleic acid an intermediate monobasic acid, $C_8H_{14}O_4$, was obtained; from its formula this was regarded as a ketohydroxy-acid, though it was not found possible to isolate from it any well-characterised crystalline derivative. The possibility of it being an unsaturated dihydroxy-acid was not excluded and in view of our present results seems probable.

The absence of oxalic or dibasic acids other than succinic acid as intermediate products differentiates the action of hydrogen dioxide from that of other oxidising agents upon palmitic acid. The introduction of the cupric catalyst converts a process of ketonisation into one which appears to consist essentially of hydroxylation and desaturation. At the same time the breaking down of the original acid is enormously facilitated.

Traube [1905; 1906; 1910; 1911; 1932] showed that the catalytic effect of cupric salts on the oxidation of the sugars and phenols was connected with the presence of the hydroxyl group in the molecule, an observation borne out by the extraordinarily rapid oxidation of tartaric acid produced by the dioxide in the presence of a cupric salt [Battie and Smedley-MacLean, 1929].

In the present investigation, the products obtained by continuous extraction with ether of the oxidation product of palmitic acid appeared to be mixtures of isomeric substances in which unsaturated dihydroxy-acids and hydroxylactones predominate. If ketohydroxy-acids are present, they occur only in small quantity and are not the main products of the reaction.

The occurrence of unsaturated acids as oxidation products of the normal higher fatty acids does not appear to have been previously observed. Ricinoleic acid, an unsaturated monohydroxy-acid, is a constituent of castor oil and its presence shows that such an acid may be a normal product of fat metabolism, but whether the ethylene linkage arises by hydroxylation and subsequent loss of water or by dehydrogenation is not known.

From the composition of the products of successive degrees of oxidation, the hydroxyl groups in these acids are probably not adjacent but occur at intervals: their position has however not yet been determined with certainty. From a comparison of the relative numbers of the C and H atoms, as determined by analysis, with the iodine values recorded, which were always lower than the expected values, it is probable that a considerable proportion of the $\alpha\beta$-unsaturated acid was present. Crotonic and cinnamic acids, for instance, give very low iodine values [Smedley-MacLean and Thomas, 1921]. It is possible that β-hydroxylation had taken place with subsequent elimination of water giving a mixture of $\alpha\beta$- and $\beta\gamma$-unsaturated acids.

The formation of a considerable proportion of unsaturated acids as intermediate products during the oxidation of the fatty acids may indicate on the other hand a process of oxygenation which is in part at any rate one of dehydrogenation, and which may be compared with the oxidation of succinic acid in the body brought about by the succinic dehydrogenase: the succinic acid is dehydrogenated forming fumaric acid and the latter adds on water giving malic acid. It is possible that the introduction of the hydroxyl group into the palmitic chain in the presence of copper activates the hydrogen atoms attached to the neighbouring carbon atoms so that they may be removed by the action of the hydrogen dioxide. Dakin has shown that in the body cinnamic acid can arise from phenylpropionic acid and can be converted into γ-phenyl-β-hydroxypropionic acid [Dakin, 1908, 2; 1909], and that the unsaturated and saturated acids gave rise to the same product. The formation of the highly unsaturated acids containing 20 and 22 carbon atoms is also known to be effected by the liver: the identification of unsaturated acids among the oxidation products of palmitic acid in the laboratory appears to offer therefore a better analogy with the method of oxidation used in the body than does the intermediate occurrence of keto-acids.

The isolation of lactones in considerable amount showed that oxidation had taken place in the γ- or δ-position.

Succinic acid was identified at every stage of the oxidation except the earliest. This acid would itself be oxidised to a considerable extent under the conditions of the experiment, but even allowing for this the total proportion of succinic acid formed would correspond to considerably less than one molecule of succinic acid for each molecule of palmitic acid oxidised. On the whole we think that the amounts formed do not support the view that molecules of succinic acid were successively split off as groups of four carbon atoms from the palmitic chain after oxidation at the γ- or δ-carbon atom [*cf.* Clutterbuck, 1927] but rather that the four terminal carbon atoms had been oxidised to succinic acid.

Volatile acids of low molecular weight were formed including propionic, acetic and formic acids and lactones containing from four to seven carbon atoms; propaldehyde and acetaldehyde were identified and propionic acid as an oxidation product of the volatile lactones. The results obtained appeared to support the view that volatile fatty acids of low molecular weight and their hydroxy-derivatives were split off from the long carbon chain.

Whatever may be the mechanism involved, oxidation by means of hydrogen dioxide in the presence of a cupric salt through intermediate stages of desaturation and hydroxylation certainly appears to be the most effective laboratory process by which the higher fatty acids may be broken down to carbonic acid and the lower fatty acids, and suggests that some such process may be involved in oxidation *in vivo*.

Method of experiment.

2 g. palmitic acid were heated at 90° with the calculated amount of *N* NaOH solution and with 15 ml. of a 2·73 % solution of crystallised copper sulphate, 212 ml. water and such an amount of *N* NaOH as was found necessary to neutralise the amount of 20 vol. H_2O_2 solution to be added gradually during the experiment. The flask was fitted with a dropping funnel and with a reflux condenser from which a tube led to a series of flasks containing *N*/2 baryta solution. At the end of the specified time, an amount of *N* sulphuric acid exactly equivalent to the *N* NaOH originally taken was added and the flask cooled,

whilst air freed from carbon dioxide was drawn through the apparatus. After filtering off any insoluble residue a measured quantity of *N* alkali was added to destroy the dioxide, and when this had been accomplished, the equivalent quantity of *N* H_2SO_4 was added, the liquid concentrated either by steam-distillation or *in vacuo* and the residual liquid continuously extracted with ether. The distillate was neutralised with *N* NaOH, the first part of the neutral distillate collected and examined for neutral substances and the residue taken to dryness. The average molecular weight of the volatile acids was calculated from the amount of *N* NaOH solution used for neutralisation and the weight of dry sodium salts.

Experimental results.

In a series of preliminary experiments, the reaction mixture was heated at 60° for 3½ hours with a large excess of hydrogen dioxide: about 80 % of the original palmitic acid was recovered unchanged; the 20 % which had disappeared had been converted into carbonic acid (8 % total carbon), volatile acids (8 %) and the remainder into a mixture of ether-soluble substances from which succinic acid was isolated. There were indications of the presence of an acid of high molecular weight giving a crystalline dinitrophenylhydrazone of unsharp melting-point and also of reducing substances. A water-soluble acid was present giving the iodoform reaction in the cold. There was however no evidence of the presence of methyltridecylketone such as would have been expected if β-keto-oxidation

Table I.

2 g. palmitic acid heated with H_2O_2 and Cu salt at 90°.

	Minutes heated	ml. 6 % H_2O_2	Wt. Cu palmitate and palmitic acid g.	Total wt. CO_2 g.	Wt. Na salt of volatile acids g.	Av. mol. wt. of volatile acids g.	% volatile reducing acid calculated as formic*	Total wt. of ether extract g.	Wt. of succinic acid g.
			Volatile acids separated by steam-distillation.						
A.	11	50	1·29	0·37	0·28	75	10·1	0·44	Negative
	20	75	1·34	0·37	0·28	75	10·1	0·44	Negative
B.	20	110	0·40	0·42	0·90	84	12·75	1·25	Positive
	15	80	0·23	0·47	0·85	78	12·29	>0·55	Positive
C.	60	175	0·21	0·68	1·02	74	18·6	1·6	0·1
	60	175	0·04	0·89	1·11	74	18·3	1·22	0·1
	60	200	0·30	0·79	1·08	63	13·15	1·21	—
	60	200	—	1·21	1·31	56	15·3	1·34	—
			Volatile acids separated by concentration *in vacuo*.						
D.	60†	175	0·16	—	0·33	67	23·8	{0·1 neutral 0·8 acid}	0·1
	60	175	0·29	—	0·52	68	34·0	1·2	0·1
	60†	175	0·30	—	0·47	—	33·0	1·0	0·05
	60†	175	0·32	—	0·60	73	—	0·81	0·11
	60	175	0·19	—	—	51	24·3	—	—
	60†	175	0·25	—	1·25	131	10·7	0·90	>0·1
	60	175	0·15	0·72	0·43	64	—	—	—
			Volatile acids separated by steam-distillation.						
E.	120	185	0·40	1·49	1·20	64	14·0	1·2	—
	120	285	—	1·49	1·20	54	5·6	—	—
F.	240	285	0·10	2·81	1·30	117	3·5	0·30	—
	240	285	0·0	2·9	1·08	53	3·8	0·17	—

* The reducing power was estimated by the reduction of mercuric chloride to calomel and calculated for purposes of comparison, but since formic acid is largely converted into CO_2 under the conditions of the experiment, other volatile reducing acids may have been present.

† In each of these experiments larger quantities of palmitic acid were oxidised and the amount of product calculated as derived from 2 g. of palmitic acid.

had taken place, as is known in the absence of the cupric catalyst. The further experiments were carried out at 90° at which temperature practically all the palmitic acid was oxidised.

After heating for 10–20 minutes at 90° with a limited amount of hydrogen dioxide, carbonic acid was evolved, and if the experiment was stopped while a considerable amount of the bluish insoluble precipitate which had separated remained, about 2/3 of the original palmitic acid was recovered, part of it as copper palmitate. About 7 % of the total carbon had been converted into carbonic acid, about 5 % into volatile acids and the remaining 8 % was present in the ethereal extract. The residue from the latter was neutralised and the solution extracted with ether to remove any neutral substances. The oil obtained from this neutral fraction contained 66·78 % C and 10·14 % H. It appeared to be a mixture of lactones corresponding approximately with the formula $C_8H_{14}O_2$ which requires C, 67·61; H, 9·86 %.

The behaviour on titration of the oil obtained from the acid fraction after it had been dried to constant weight in a vacuum-desiccator suggested the presence of a lactonic acid, since the colour of the indicator repeatedly disappeared on warming with additional quantities of $N/10$ NaOH. (Found for the free acid (micro-analysis) C, 64·13; H, 9·64 %. Mol. wt. by titration, 224 to 260; from analysis of Ag salt, 244. $C_{13}H_{24}O_4$ requires C, 63·93; H, 9·84 %. Mol. wt. 244.) Attempts to prepare a crystalline derivative from this acid were unsuccessful, and no evidence of the presence of the keto-group was obtained. A reducing group was present, since when warmed for a considerable time with ammoniacal silver nitrate solution a good silver mirror was deposited.

When the acid was warmed for one hour on a boiling water-bath with 50 % sulphuric acid, a strong gorse-like smell, characteristic of fatty lactones was developed, and a very small amount of a neutral substance containing 70·40 % C and 10·40 % H was isolated agreeing with the theoretical values for the lactone $C_{10}H_{18}O_2$ (70·59 % C; 10·59 % H).

From its behaviour on titration it appeared that the fraction having the composition $C_{13}H_{24}O_4$ contained a certain percentage of lactone but that most of it consisted of free acid, the proportions of carbon and hydrogen agreeing with those required by a ketohydroxy-acid. Since however the keto-group was not detected and could certainly not have been there to any large extent, it seemed probable that the bulk of this product was an unsaturated dihydroxy-acid. The substance reduced permanganate in neutral solution. At this stage no succinic acid was detected.

The formation of a product agreeing with the composition $C_{13}H_{24}O_4$ makes it probable that not only had γ- or δ-hydroxylation of the original C_{16} acid taken place but that hydroxylation had also taken place at two or three other points of the chain. The unsaturated linkage present in the C_{13} product may indicate that hydroxylation of the atom in the β-position to the 13th carbon atom had occurred with subsequent elimination of water, and the presence of a lactone that either the γ- or δ-carbon atom had been hydroxylated. Since, after heating with sulphuric acid, the lactone isolated had the composition $C_{10}H_{18}O_2$, it is possible that the remaining OH-group was in the γ- or δ-position to the 13th carbon atom.

When the proportion of hydrogen dioxide was increased, a point was reached at which rapid solution of nearly all the precipitate floating on the surface of the reaction mixture occurred, and practically the whole of the palmitic acid had been converted into water-soluble products. At this stage the quantity of carbonic acid given off was only slightly increased, but the amount of volatile

acids separating on steam-distillation was very much greater, and the average molecular weight showed that acids of higher molecular weight than acetic acid were present (*cf.* Table I). The residue from the ether extract was much increased, and its behaviour on titration again showed that a lactonic acid was present. Crystals of succinic acid were isolated and identified by a mixed melting-point.

When the reaction product was heated with a considerable excess of hydrogen dioxide for one hour, the quantities of carbonic and volatile acids produced were increased, but the average molecular weight of the latter was diminished. When the product was concentrated by distillation *in vacuo*, the total weight of volatile acids was less than half of that obtained when the volatile acids were separated by steam-distillation, and the proportion of a reducing volatile acid was much higher. The ether extract of the product obtained after the residue had been concentrated *in vacuo* smelt of acetic acid and only reached constant weight after long evacuation in a vacuum desiccator. The difference shown in the amount of volatile acids obtained by the two different methods of working up (*cf.* Table I) may be reckoned as due to the incomplete separation of the volatile acids, since during the concentration *in vacuo*, the product was not taken to complete dryness. In one experiment, the volatile distillate was neutralised with NaOH solution and extracted with ether. (Found (micro-analysis) C, 54·71; H, 8·97 %. $C_6H_{12}O_3$ requires C, 54·54; H, 9·09 %.) The amount was very small and no evidence as to structure was obtained.

In another experiment the sodium salts of the volatile acids were treated with strong H_2SO_4, and the diluted product was extracted with ether. The ethereal layer was shaken with potassium carbonate solution and the alkaline aqueous layer acidified and extracted with ether. From this ethereal solution an oil was obtained. (Found C, 43·99; H, 1·63 %. Mol. wt., by titration, 108; by analysis of Ag salt, 95. $C_4H_8O_3$ requires C, 46·15; H, 1·69 %. Mol. wt. 104.) From the neutral ethereal layer separated from the potassium carbonate solution, a substance of characteristic lactone odour was separated. (Found C, 64·53; H, 9·81 %. $C_5H_{10}O_2$ requires C, 63·16; H, 8·77 %. $C_7H_{12}O_2$ requires C, 65·63; H, 9·38 %.) Volatile hydroxy-acids and lactones had therefore been formed as decomposition products.

In the neutral volatile portion (obtained by redistilling the neutralised distillate, which had been separated either by steam-distillation or by concentration under diminished pressure), propaldehyde was identified by means of its orange-red dinitrophenylhydrazone, melting after recrystallisation from alcohol at 153–154° and the result was confirmed by analysis of the hydrazone. (Found N, 24·05 %. $C_9H_8N_4O_4$ requires N, 23·73 %.)

After oxidising the volatile acids with chromic acid, a neutral product giving analytical figures between those required for the lactones $C_6H_{10}O_2$ and $C_7H_{12}O_2$ was obtained. When the sodium salts of the volatile acids were treated with strong sulphuric acid and the product distilled, propionic acid was identified in the distillate and separated as the characteristic basic lead salt. The routine method for estimating formic acid by reduction of mercuric chloride was also applied to the mixture of volatile acids and very considerable reduction took place. Since however formic acid is readily destroyed under the conditions of the experiment, some other reducing volatile acid may have been present.

After one hour's heating, for every 2 g. of palmitic acid oxidised, between 0·1 and 0·2 g. succinic acid was isolated as crystals from the mixture, but there was evidence that considerably more was formed.

The residue from the ethereal extract was divided into two parts by means

of its solubility in benzene. In one experiment in which 24 g. of palmitic acid had been oxidised, 6·72 g. of an oil insoluble in benzene and 4·6 g. of one soluble in benzene were obtained.

From the oil insoluble in benzene more than 1 g. of succinic acid separated as crystals and the residue was divided into two fractions: (1) a part soluble in water of low average molecular weight (94 by silver salt analysis, 122 by titration) containing a considerable amount of succinic acid and giving a silver salt which rapidly darkened; (2) a part insoluble in water. The latter had an average molecular weight of 167, determined by titration and apparently contained about 20 % lactone. (Found C, 53·74; H, 7·87 %.) Its iodine value was 22·4. The simplest formula agreed most nearly with $C_7H_{12}O_4$, which requires C, 52·50; H, 7·50 %. Since the proportion of carbonyl group determined by its reaction with sulphite was negligible, and considering the proportion of carbon to hydrogen atoms, unsaturated hydroxy-acids must have been present with a small proportion of hydroxylactone.

The composition of the water-soluble fraction (1) varied in different experiments from C, 52·7 to 56·5; H, 7·1 to 7·5 %, and it contained an appreciable amount of succinic acid. Estimation of the carbonyl group showed that less than 2 % was present; the iodine value was 35, indicating a considerable proportion of unsaturated acid in the mixture.

The composition of the fraction soluble in benzene varied in a number of different experiments from 58·44 to 65·69 % C and from 7·8 to 9·7 % H. The molecular weight determined by titration gave values from 150 to 200 and the product contained about 21 % lactone. The product was divided into neutral and acid fractions.

The neutral product appeared to consist of a mixture of hydroxylactones for the separation of which neither distillation nor fractionation by means of solvents has yet given satisfactory results. A fraction insoluble in light petroleum contained 63·00 % C and 8·08 % H. Its molecular weight (determined by heating a known weight for one hour on a water-bath dissolved in alcohol with a known amount of NaOH and titrating the excess) gave a molecular weight of 174, but the end-point was not very satisfactory. This substance did not react with a solution of sodium bisulphite and was therefore free from any appreciable amount of keto-compound. Its iodine value was 68, corresponding with the existence of rather less than half an ethylene linkage for the observed molecular weight. As already pointed out an ethylene linkage in the $\alpha\beta$-position gives a very low iodine value. The available evidence would point to the presence of the lactone of an unsaturated hydroxy-acid corresponding approximately with the formula $C_9H_{14}O_3$, which requires C, 63·53; H, 8·23 %. Mol. wt. 170. Until a better method of separation and identification has been found a definite formula cannot be ascribed to it.

The acid fraction was converted into sodium salts, the solution of which was evaporated to dryness and the dry salts fractionated by their solubility in 96 % alcohol and acetone. The acid derived from a very hygroscopic sodium salt soluble in alcohol and not precipitated from alcoholic solution by the addition of acetone agreed in composition with the formula $C_{10}H_{18}O_4$. (Found C, 59·58; H, 8·39 %. Mol. wt. by titration 200, 203. $C_{10}H_{18}O_4$ requires C, 59·40; H, 8·91 %. Mol. wt. 202.) It no longer reacted as a lactonic acid, and the estimation of the carbonyl group was negative. The I.V. was 16·5. It may therefore be regarded as a dihydroxydecenoic acid.

For purposes of reference we synthesised the α-keto-derivatives of octanoic, nonanoic and decanoic acids and examined their properties. The method adopted

was that used by Bouveault and Locquin [1904], in which nitrosylsulphuric acid was allowed to act upon the ethyl ester of the α-acetyl derivative of the normal fatty acid in the presence of concentrated sulphuric acid, when the acetyl group was replaced by the group —NOH. By the further action of nitrosylsulphuric acid in the presence of formic acid the corresponding α-keto-acid was obtained.

Preparation and properties of α-keto-octanoic acid.

The ethyl ester of α-acetyloctanoic acid was prepared by heating together equimolecular proportions of sodium ethoxide, ethyl acetoacetate and hexyl iodide. The oxime of this ester (M.P. 52–53°) was prepared and, after recrystallisation, converted into α-keto-octanoic acid [Bouveault and Locquin, 1904; Blaise and Luttringer, 1905]. This was a colourless crystalline substance, smelling strongly of "goat," which melted at 117–118° after recrystallisation from alcohol and subsequent washing with light petroleum, in which it was sparingly soluble. It formed an oxime, M.P. 110°, and a bright yellow dinitrophenylhydrazone which, after two recrystallisations from alcohol, melted at 136·5–137·5°. The acid gave a silver mirror on warming with ammoniacal silver nitrate. (Found: C, 60·28; H, 8·76 %. Theoretical: C, 60·76; H, 8·86 %.)

Preparation and properties of α-ketononanoic and α-ketodecanoic acids.

These acids were prepared in a similar manner. α-Ketononanoic acid was derived from the ethyl ester of α-acetylnonanoic acid [Jourdan, 1880]; the oxime was a white crystalline substance melting at 58–59° after recrystallisation from dilute acetic acid. α-Ketononanoic acid melted at 105–106°, formed an oxime melting at 112°, and a dinitrophenylhydrazone melting at 129°. It reduced ammoniacal silver nitrate. (Found: C, 62·26; H, 9·08 %. Theoretical: C, 62·78; H, 9·30 %.) α-Ketodecanoic acid was prepared from ethyl α-acetyldecanoate [Guthzeit, 1880]; the oxime melted at 60–61°. The α-keto-acid melted at 85–86° after recrystallisation from light petroleum. It gave a dinitrophenylhydrazone, M.P. 126–128°, and a silver mirror with ammoniacal silver nitrate. (Found: C, 64·16; H, 10·15 %. Theoretical: C, 64·51; H, 9·68 %.)

SUMMARY.

Although we have not yet been able to characterise substances of undoubted chemical individuality from the complex mixture of ether-soluble products obtained on oxidising palmitic acid by means of hydrogen dioxide in the presence of a cupric salt, certain inferences as to the nature of the products and therefore as to the intermediate stages of the process can be made.

1. Hydroxylation appeared to take place simultaneously at several points of the palmitic chain.

2. The palmitic carbon chain was gradually broken down, and evidence of the formation of unsaturated hydroxy-acids containing ten carbon atoms was obtained. There was also some indication that chains containing respectively thirteen and seven carbon atoms were formed during the reaction, but the chemical individuality of these substances requires further confirmation.

3. Ketonic acids were present, if at all, only in very small quantities and were certainly not the main product of oxidation.

4. Lactones, indicating the formation of γ- or δ-hydroxy-acids were split off during the course of the reaction.

5. Unsaturated hydroxy-acids were present and, from the analyses of the products, these formed the main bulk of the intermediate substances. The low

iodine values recorded probably indicated the presence of some unsaturation in the 1:2-position.

6. Volatile acids of low molecular weight were formed in considerable quantity and were split off at an early stage.

7. When heated for 4 hours at 90° more than 50 % of the total carbon of the palmitic acid appeared as CO_2 and more than 20 % as volatile acids.

8. For purposes of reference the α-ketoheptanoic and α-keto-octanoic acids were prepared and characterised.

The study of these intermediate compounds is in progress and the result will form the subject of a further communication.

The micro-analyses were carried out in Dr Schoeller's laboratory. We desire to acknowledge the assistance given by a grant from the Department of Scientific and Industrial Research.

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LXXI. NOTE ON THE STORAGE OF CARBOHYDRATE AND FAT BY *SACCHAROMYCES FROHBERG* WHEN INCUBATED IN SUGAR SOLUTIONS.

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(*Received February 19th, 1934.*)

The storage of carbohydrate and fat by yeast on incubation in various sugar solutions (glucose, fructose, sucrose, maltose, lactose) was studied by Smedley-MacLean and Hoffert [1923; 1924]; the investigation has now been extended to include galactose.

The brewery yeast used in the previous experiments, a mild ale yeast which did not ferment galactose, produced on incubation in a 4 % solution of this sugar no increase in fat and a fall in carbohydrate. *Saccharomyces Frohberg*, a yeast known to ferment galactose after acclimatisation, was therefore tried. This yeast was acclimatised to galactose by culturing on a hydrolysed lactose and yeast extract medium, and grown, at laboratory temperature, on a 5 % sucrose and yeast extract medium, 4 litres of which furnished about 20 g. of yeast after a week's growth.

6·25 g. of the *Frohberg* yeast were analysed as a control, and two portions of 6·25 g. incubated in 500 ml. of each of the two solutions to be compared, for 44 hours, with oxygenation, at room temperature (*ca.* 15°).

The methods of estimation of carbohydrate and fat were those described in the previous communication.

Possibly owing to the different media on which they had been grown, the carbohydrate content of the *Frohberg* was somewhat lower than that of the brewery yeast, while the fat content of the former was nearly twice as great.

The storage in 2, 4 and 5 % glucose and galactose solutions was studied, equal portions of the same sample of yeast being incubated, under the same conditions, in the two solutions to be compared. An increase of carbohydrate storage and of fat storage with increasing concentration of sugar was shown both in glucose and galactose solutions. There was no significant difference between the amount of carbohydrate stored on incubation in the same concentration of glucose or galactose; in glucose, however, more fat was stored at all concentrations than in galactose (see Figs. 1 and 2).

The effect of the presence of phosphate (0·49 g. $Na_2HPO_4, 12H_2O + 0·143$ g. KH_2PO_4 per 500 ml. of solution, p_H about 7·8) in 5 % solutions of glucose and galactose was then observed. It was shown previously that addition of phosphate to solutions of glucose, fructose, sucrose and maltose, brought about an increased storage of fat and also of phosphorus in brewery yeast incubated therein. This effect was marked and led to the suggestion that fat formation in yeast proceeded *via* hexosephosphate. The *Frohberg* yeast behaved differently. When it was incubated in a 5 % glucose solution to which phosphate had been

added there was no increase in fat storage, the variation was rather a decrease but not of significant amount; the presence of phosphate in a 5 % galactose solution caused a slight increase in fat, but this was not of the same order as the increases referred to above.

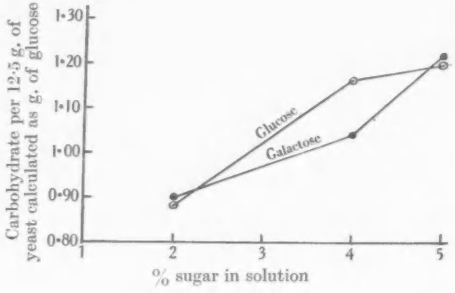

Fig. 1.

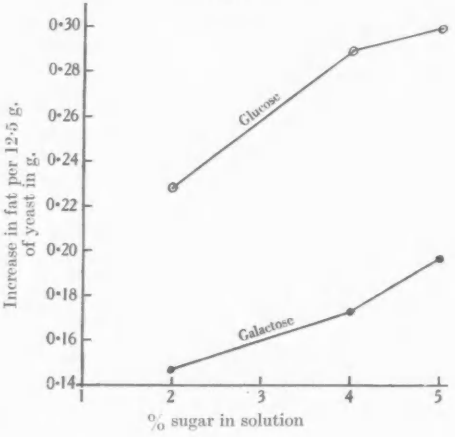

Fig. 2.

Estimation of phosphorus content, by Neumann's method, showed no significant variations.

A somewhat more marked effect seems to be shown on carbohydrate storage. The addition of phosphate to a glucose solution caused a diminution, to a galactose solution an increase, in carbohydrate content.

Estimation of the residual sugar in a solution in which yeast had been incubated, showed that in parallel experiments, nearly twice as much glucose was used up as galactose. Tests were performed to ascertain whether the *Frohberg* yeast lost its power of fermenting galactose when grown on sucrose media and the results indicated that this was so; since the amount of galactose (up to 10 g.) used up during a 44-hour incubation could not, however, have been accounted for by increased storage within the cell and some fermentation of galactose must have taken place though this was probably retarded. There was no

Table I.

The numbers represent g. per 12·5 g.* original yeast.

% sugar in medium		Original yeast		After incubation in glucose solution		After incubation in glucose + phosphate solution		After incubation in galactose solution		After incubation in galactose + phosphate solution		After incubation in maltose solution		After incubation in fructose solution		After incubation in sucrose solution	
		Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.	Carbo-hydrate g.	Fat g.
2		0·72	0·172	1·00	0·406	—	—	0·88	0·288	—	—	—	—	—	—	—	—
		0·82	0·166	0·76	0·414	—	—	0·88	0·314	—	—	—	—	—	—	—	—
		1·00	0·144	0·84	0·374	—	—	0·96	0·330	—	—	—	—	—	—	—	—
		0·98	0·128	0·92	0·376	—	—	—	—	—	—	—	—	—	—	—	—
	Mean	0·88	0·152	0·88	0·392	—	—	0·90	0·310	—	—	—	—	—	—	—	—
4		0·70	0·178	1·10	0·496	—	—	1·03	0·364	—	—	—	—	—	—	—	—
		0·63	0·156	1·22	0·410	—	—	1·07	0·310	—	—	—	—	—	—	—	—
		—	—	1·13	0·450	—	—	—	—	—	—	—	—	—	—	—	—
	Mean	0·66	0·166	1·16	0·454	—	—	1·04	0·336	—	—	—	—	—	—	—	—
5		0·96	0·238	1·41	0·552	0·96	0·386	1·46	0·406	1·74	0·508	0·90	0·250	1·16	0·380	1·02	0·370
		1·06	0·150	1·00	0·492	0·78	0·482	1·14	0·366	0·88	0·338	1·33	0·294	0·98	0·346	1·24	0·348
		0·93	0·182	1·12	0·482	0·90	0·366	1·34	0·370	1·20	0·404	—	—	—	—	—	—
		0·84	0·166	1·27	0·366	0·82	0·368	1·08	0·338	1·50	0·348	—	—	—	—	—	—
		0·82	0·158	—	—	—	—	1·05	0·316	—	—	—	—	—	—	—	—
		0·74	0·158	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·94	0·180	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·96	0·194	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·94	0·180	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		1·02	0·152	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·67	0·158	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		0·71	0·138	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Mean	0·84	0·160	1·20	0·464	0·86	0·400	1·22	0·360	1·32	0·400	1·11	0·272	1·07	0·363	1·13	0·359

Milligrams phosphorus per 12·5 g. of original yeast in g.

		Mean
Original yeast	72, 66, 68, 76, 98	76
After incubation in 5 % glucose solution	74, 44 — — —	60
After incubation in 5 % glucose + phosphate solution	64, 98, 88, 106 —	88
After incubation in 5 % galactose solution	80, 56, 84 — —	74
After incubation in 5 % galactose + phosphate solution	84, 70, 66, 54 —	68

* Results are given per 12·5 g. of yeast for comparison with previous work on brewery yeast.

difference between the amount of sugar used up from a sugar solution and from a similar solution with phosphate.

The *Frohberg* yeast was also incubated in 5 % solutions of maltose, fructose, and sucrose. The carbohydrate storage, in these sugars, was of the same order as in glucose and galactose at the same concentration; particularly remarkable was the total absence of the extra carbohydrate storage shown by brewery yeast in maltose, attributed then to the possible direct formation of glycogen from maltose. Fat storage was poorer in maltose solution than in galactose and in fructose and sucrose of the same order as in galactose; it was in no case so good as in glucose.

SUMMARY.

1. *Carbohydrate storage.* The carbohydrate storage of *Saccharomyces Frohberg* on incubation in solutions of glucose, galactose, fructose, sucrose and maltose of the same strength is similar in all cases. Glucose and galactose were investigated in varying concentration whereby it was shown that there was an increased storage of carbohydrate with increased concentration of either sugar in the medium.

Frohberg yeast behaved similarly to brewery yeast when incubated in solutions of glucose, galactose, fructose and sucrose but showed a marked difference on incubation in maltose. While *Frohberg* yeast stored normal amounts of carbohydrate in the case of this sugar, in brewery yeast an abnormally high carbohydrate storage occurred.

2. *Fat storage. Frohberg* yeast showed a greater power of forming fat when incubated in solutions of glucose than in those of galactose, fructose, sucrose, or maltose; the last seemed the least effective. In the cases of glucose and galactose the amount of fat stored increased with increase of sugar in the surrounding medium. The addition of phosphate to the medium did not appear to cause any such increase of fat storage as was previously noted in the case of brewery yeast.

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LXXII. THE OSMOTIC PRESSURE OF GUM ACACIA SOLUTIONS.

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(*Received February 13th, 1934.*)

The great and increasing use of solutions of gum acacia, with or without the addition of sodium chloride, as an infusion in cases of shock and loss of blood [Bayliss, 1918], together with the mass of data now available relating to the osmotic pressure of the blood-colloids in health and disease, render an exact knowledge of the osmotic pressure of the colloids in gum acacia solutions of importance.

The osmotic pressure of the solution is likely to be modified by such factors as the concentration of salts present, the heat treatment to which the solution has been submitted for purposes of sterilisation and, perhaps, the dilution of the gum, a concentrated solution containing larger complexes than one more dilute.

It was, therefore, decided to study the effects of concentration, of the addition of salts and of the influence, if any, of different methods of sterilisation upon the osmotic pressure of the colloids in solutions of gum acacia.

Methods.

Apparatus. For the majority of the experiments recorded below the Krogh [Krogh and Nakazawa, 1927] type of osmometer, made by the Zsigmondy Ultrafilter company, was used. In a few of them the osmometer used by Widdowson [1933] was employed. The osmometers were set up in all the three possible positions—dialysate above plate with bent capillary; dialysate below plate (Krogh); dialysate above plate with straight capillary—and, as concordant results were obtained with all of these, the pressures measured were obviously real. A loose-fitting cap containing damp cotton wool covered the open ends of the osmometers in cases one and three above, while the end dipped into water when in the "Krogh" position. Evaporation of the dialysate was thus prevented. Pressures were recorded in cm. of water.

Membranes. In the present series of experiments only cellophane ("No. 600") as used by Widdowson and by Govaerts [1927] was employed.

Time and temperature. Between 22 and 24 hours was allowed for equilibrium to be attained. 17 hours was definitely too short a time and in several cases good readings were obtained after a lapse of 46 hours. One of the osmometers was accommodated in a vacuum flask, but this did not seem to add much to the consistency of the readings. All pressures were reduced to 0°.

Material. Finest commercial "Gum acacia parv" was used for making the test solutions. Solutions were never more than 3 days old for "fresh" gum.

When sterilised material was employed it was sterilised in small bottles and a fresh one used if the previous one had been open for more than 3 days. Some fresh

gum solutions were preserved by the addition of sufficient thymol to saturate the mixture.

The standard infusion solution used clinically contains 6 % of gum acacia. This strength was made a standard and all results referred to it.

Effect of concentration on osmotic pressure.

Solutions of varying strength were examined. It was not possible, with the apparatus in use, to measure pressures much higher than were given by a solution of 6 % concentration.

Table I.

Strength of solution %	Osmotic pressure observed cm. water at 0°	Osmotic pressure for 6 %
2	28·1	84·3
2	23·9	71·7
3	37·4	74·8
3	40·4	80·8
3	40·0	80·0
3	38·8	77·2
6	80·8	80·8
6	77·7	77·7

Mean value (for 6 % gum) 78·4 cm. p_H 4·4–4·5.

The results in Table I show that the osmotic pressure is not affected within the limits of experimental error by alteration in the strength of the solution between the limits 2–6 %. For solutions saturated with thymol the results are given in Table II.

Table II.

Strength of solution %	Osmotic pressure observed cm. water	Osmotic pressure for 6 % at 0°
2	23·82	71·46
2	25·55	76·65
3	40·1	80·2
3	36·5	73·0
4	48·9	73·3
6	73·4	73·4

Mean value (for 6 % gum) 74·7 cm.

Again it is obvious that dilution has not affected the observed osmotic pressure. The presence of thymol causes a diminution of 4·9 % in the osmotic pressure.

Effect of the addition of sodium chloride (Table III).

Solutions containing 6 % of gum and 0·9 % salt (corresponding to the clinical solution of 6 % gum in normal saline) were examined.

Mean osmotic pressure observed 11·93 cm.; p_H 4·2.

Table III. *Effect of different concentrations of sodium chloride.*

Gum 6 % containing salt (%)	Mean osmotic pressure	p_H
2·0	11·0	—
0·9	11·93	4·2
0·46	15·8	4·3
0·31	18·0	—
0·276	18·26	4·4
0·2	21·6	—
0·1	32·6	—
0·05	49·5	—
Nil	78·4	4·5

The addition of salt has therefore a profound depressing action on the osmotic pressure of the colloids in gum solutions.

6 % gum in 0·9 % saline saturated with thymol, 11·32 cm.

Saturating the solution with thymol depresses the osmotic pressure by the same proportion (4·9 %) as was found for pure gum solutions.

The figures obtained all lie on a smooth curve of hyperbolic type, and no discontinuity is observed. (See Fig. 1.)

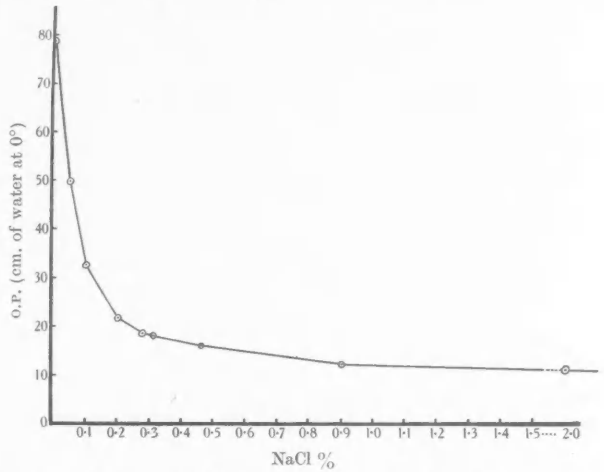

Fig. 1. Osmotic pressure of 6 % gum acacia solution with varying amounts of NaCl.

The p_H of the solution falls proportionately as the salt content rises. This is due to a change in the gum, not to the salt itself, since 3 % gum in normal saline has a p_H of 4·3 as against the value of 4·2 for 6 % gum containing the same amount of salt.

Effect of dialysis (*Table IV*).

In view of this series of results it was decided to measure the osmotic pressure of gum solutions purified from salts, naturally present in the solution, by dialysis.

Pure gum solutions were dialysed in a pair of Asheshov's tubes, fitted with a cellophane membrane, against distilled water. The gum was preserved by the addition of thymol as above and the distilled water was also saturated with this preservative. The osmotic pressures were corrected for the depressing action of thymol by the amount found to be correct from earlier work. The water was changed daily.

Table IV. *Effect of dialysis.*

	Osmotic pressure cm.
Gum solution 1 month old dialysed for 5 days gave	83·6 for 6 %
Gum dissolved in apparatus and dialysed 7 days gave	115 for 6 %
Gum freshly dissolved	78·4

The difference between the two results above may be ascribed to one of three causes or to a combination of them.

1. Thymol does not protect the gum completely against bacterial action.
2. The osmotic pressure of a gum solution may alter naturally with time.
3. The second solution was dialysed for a longer time.

It is considered that the first cause cited above is the most likely.

As expected, purification of the gum solution by dialysis caused a rise in the observed osmotic pressure.

Sterilisation.

The effect of different types of heat treatment on the osmotic pressure was investigated on four solutions containing 6 % of gum. The treatments to which these were subjected were as follows (Table V).

Table V. *Effect of heat treatment.*

Solution A, steamed for 20 minutes on 3 successive days; B, autoclaved to 30 lbs. pressure; C, autoclaved to 30 lbs. pressure and then to 7 lbs. for 10 mins.; D autoclaved to 30 lbs. pressure, and then steamed on 3 successive days.

The osmotic pressures are those for 6 % concentration.

Solution	Mean osmotic pressure observed cm. water	p_H
A	80·9	4·55
B	77·3	4·65
C	98·2	4·55
D	101·6	4·60
Fresh gum	78·4	4·4–4·5

For gum solutions containing 0·9 % of sodium chloride the values in Table VI were obtained (the letters refer to the same heat treatment as in the pure gum series).

Table VI.

Solution	Mean osmotic pressure	p_H
A	12·87	4·4
B	12·59	—
C	15·91	4·3–4·2
D	17·04	4·3
Fresh gum saline	11·93	4·2

The conclusions to be drawn from these two sets of results are that, while sterilising by any method does break up the gum molecule, such damage is not so profound as to render the resulting particles dialysable to any marked extent. If this had occurred a diminution in the value of the osmotic pressure would have been found instead of the increase actually met with.

The second sterilisation (after filling into the final container) does more damage than the first and steaming on 3 successive days is more active as a disruptive agent than is the autoclave treatment.

The same order of increase in osmotic pressure is noted in both sets of solutions, but the proportionate increase is greater when salt is present (Table VII).

Table VII

Solution	Saline osmotic pressure	Pure (calc. from saline figures) osmotic pressure	Pure (observed) osmotic pressure
Fresh	11·98	(78·4)	(78·4)
A	12·87	85·3	80·9
B	12·59	83·5	77·3
C	15·91	105·5	89·2
D	17·04	112	101·6

The p_H of gum solutions is not markedly affected by sterilisation.

Discussion and summary.

The results detailed above show that while the colloid osmotic pressure of gum acacia is independent of the concentration over the range 2–6 %, it is greatly reduced by the presence of salt. The actual osmotic pressure of 6 % gum in normal saline is only little more than one-third of that of the blood-colloids, and, while sterilisation causes a considerable increase in the osmotic pressure, the latter is even then less than one-half that of the colloids in normal blood. It would seem that the addition of salt to gum solutions is, partially at least, defeating the clinical object of the administration of such treatment.

The sterilised gum solutions used in the work were supplied by The Crookes Laboratories.

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LXXIII. THE CHEMISTRY OF MOULD TISSUE[1].

V. FRACTIONATION OF THE NITROGEN IN THE MYCELIUM OF *ASPERGILLUS FISCHERI.*

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(*Received February 17th, 1934.*)

In view of the enormous number of mould species and the high nitrogen content of the mycelium (4–10 %), it is surprising to find how meagre are the data on the nitrogenous constituents of such tissues. A review of the literature shows that only four or five species have been investigated and most of these only casually.

Small quantities of alkali-soluble proteins have been isolated by several investigators [Thomas and Moran, 1914; Takata, 1929, 2; Thomas, 1928; 1930; Clutterbuck *et al.*, 1932]. Such proteins have been uniformly low in nitrogen (12–13 %); some have been high in phosphorus (0·7 %); and some have given strong tests for carbohydrate. On the basis of such data Takata [1929, 1] suggested that a protein which he isolated might be regarded as a phosphoglycoprotein. Certain it is that the proteins of the mould pad are difficult to isolate and appear to be different in character from most of the proteins of plant and animal materials.

A number of amino-acids have been identified in the acid hydrolysate of the mycelium or proteins separated therefrom. Glycine, alanine, leucine, tyrosine, lysine, arginine, histidine, tryptophan, aspartic and glutamic acids have been reported by one or more investigators [Takata, 1929, 2; Clutterbuck *et al.*, 1932; Abderhalden and Rona, 1905; Vorbrodt, 1919; 1921]. As shown by Abderhalden and Rona [1905], the mould, *A. niger*, was able to synthesise the same amino-acids from either inorganic nitrogen (*e.g.* potassium nitrate) or organic nitrogen (*e.g.* glycine or glutamic acid).

Indirect evidence from feeding experiments with animals [Takata, 1929, 1; Skinner *et al.*, 1933] indicates either the absence of certain essential amino-acids or their presence in too low a proportion to bring about growth at an intake level of 15–18 % protein. Such feeding trials also indicate considerable variation in amino-acid content among different species of moulds and it may be even in the same species under different conditions of growth.

Besides amino-acids, xanthine, hypoxanthine, guanine, adenine, cytosine, glucosamine, choline, betaine and stachydrine have been reported [Takata, 1929, 2; Vorbrodt, 1921] among the hydrolysis products of *A. niger* and *A. oryzae*. Whether or not such compounds are found generally in mould mycelium cannot be decided on the basis of the data now available.

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

In view of the incompleteness of the data, it seemed desirable (*a*) to separate the total nitrogen of mould pads into various fractions according to their solubilities and (*b*) to determine the partition of nitrogen in each of these fractions.

Experimental.

The mould tissue used in the following experiments was a pure culture of *A. fischeri* grown in batches of 25 kg. on a glucose-inorganic salts medium. The details of the procedure used in growing the mould have been reported in a previous paper [Peterson *et al.*, 1933].

Preliminary experiments.

Several preliminary nitrogen extraction experiments were conducted. While the data from these experiments cannot be presented here because of lack of space, the following statements give the more important results obtained.

Mould tissue which had been killed by steaming, then dried and ground and subjected to long extraction with hot alcohol to remove fat and sterols [Pruess *et al.*, 1932] proved to be unsatisfactory for nitrogen studies. Tissue that had been thus treated absorbed water and formed a slimy, jelly-like mass, which was not filtrable. The extract remained turbid even after passage through a Sharples super-centrifuge. The nitrogen of the mycelium distributed itself among successive solvents as follows: water, 11·7 %; 2 % NaOH, 28·2 %; 5 % NaOH, 30·8 %; alkali-insoluble, 18·9 %.

Mould tissue which was killed by steaming at 99° for one hour, then dried for 48 hours at 65° and ground was more satisfactory in that it could be filtered by draining overnight from fluted filters. The solubility of the nitrogen in this material was as follows: water, 18·9 %; 2 % NaOH, 26·7 %; 5 % NaOH, 28·7 %; alkali-insoluble, 17·1 %.

The best results were obtained when perfectly fresh untreated mycelium was used. Aqueous extracts of this material filtered readily and gave clear filtrates. However, complete extraction of water-soluble substances was obtained only after extraction of the residue with alcohol-ether following a first water extraction. When this was done, an additional quantity of water-soluble nitrogen, almost equal to that contained in the first extract, was obtained. 10 % sodium chloride solution or 1 % hydrochloric acid solution extracted about the same percentage of the total nitrogen as did water alone.

Extractions of the water-insoluble residue with alkaline (KOH, $NaHCO_3$, Na_2CO_3, $Ba(OH)_2$) solutions of various strengths and under different conditions of time and temperature were compared. 1 % KOH solution at room temperature followed by 4 % KOH solution at boiling temperature seemed to give the most regular and informative results.

On the basis of the preliminary experiments the final extraction procedure given below was adopted.

Method of extraction.

Mould tissue fresh from the pans of the steriliser-incubator was rinsed with distilled water and immediately ground to a fine paste in a "Nixtamal" mill. The ground pad was allowed to fall into a dish containing chipped ice and was stirred into the ice. When the pad had all been ground, it was emptied into a jar of iced water and stirred vigorously for 15 minutes with an electric stirrer. It was then filtered with suction on a cloth filter and the filtrate poured back upon the filter-cake and refiltered until it came through clear. The filter-cake was given two

more 15-minute extractions with iced water, and all three filtrates were combined and comprise the "First water-extract."

The filter-cake from the water-extract was thoroughly stirred into a large excess of alcohol-ether (1 : 1), and allowed to stand overnight. The alcohol-ether was filtered off, and the filter-cake was suspended in ether and allowed to stand several days before filtering. The filtrates were combined; they constitute the "Lipin fraction."

The ether-extracted filter-cake was spread out in a thin layer and air-dried before a fan for 2 hours. The dry powder was then stirred with water for 15 minutes and filtered. The filter-cake was re-suspended in water and allowed to soak overnight under protection of toluene. It was then filtered, and the two filtrates combined formed the "Second water-extract."

The residue from the water extraction was stirred into an excess of 1 % KOH solution and allowed to stand at room temperature for 2 days. The suspension was filtered with suction on a cloth filter, the filter-cake washed twice by stirring with water and filtering, and the three filtrates were combined. This is designated as "Cold 1 % KOH extract."

The filter-cake was boiled with 4 % KOH solution for one hour, filtered and washed twice with water, and the filtrates were combined to form the "Hot 4 % KOH extract."

The residue from the hot alkaline extraction was washed with 50 % alcohol, 95 % alcohol and ether and then dried. This fraction is called "Residue."

The extraction procedure as described above has been carried out a number of times, and two typical analyses (on batches 28 and 31) are presented in Table I.

Table I. *Nitrogen distribution of* A. fischeri.

	Percentage distribution				Percentage composition of fractions				Free amino-nitrogen. Percentage of total nitrogen in each fraction
	Nitrogen Batch		Solids (ash-free basis) Batch		Nitrogen (ash-free basis) Batch		Ash Batch		
Fractions	28	31	28	31	28	31	28	31	Batch 31
First water-extract	17·2	16·8	19·8	15·6	4·4	6·7	13·4	11·6	37·5
Lipins	1·2	1·2	9·5	8·5	0·9	1·1	0·0	0·0	†
Second water-extract	13·6	11·7	17·0	14·8	4·1	4·9	1·4	1·8	25·0
Cold 1 % KOH extract	39·7	41·2	21·1	22·9	9·6	11·1	35·0*	33·6*	9·4
Hot 4 % KOH extract	13·0	14·3	13·7	15·2	4·9	5·8	64·4*	54·1*	14·0
Residue	12·2	13·8	26·0	27·2	2·3	3·1	2·9	2·4	†
Total recovered	96·9	99·0	107·1	104·2	—	—	—	—	—
Whole mould	100·0	100·0	100·0	100·0	5·1	6·2	3·0	2·9	†

* Including ash from alkali. † Not determined.

Nitrogen distribution.

As can be seen from Table I, 17·2 and 16·8 % of the total nitrogen of the mould pad of batches 28 and 31, respectively, were dissolved in the first water-extract. The nitrogen content of the extracted solids in batch 28 was slightly less (4·4 %) than that of the whole mould (5·1 %), whereas in batch 31 it was slightly more (6·7 % against 6·2 %).

The first water-extract was a yellowish green solution of p_H 4·6. It gave negative biuret, Millon and Hopkins-Cole tests and a positive xanthoproteic test. It gave no precipitate with trichloroacetic, acetic or hydrochloric acid, but gave a slimy brown precipitate when made alkaline with KOH, and a further precipitate upon the addition of baryta to the filtrate from the KOH precipitation. Neither of these two precipitates contained as much as 1 % of nitrogen. The KOH precipitate contained 56 % ash and the baryta precipitate about 90 %. Since the extracted solids contained about 12 % ash, it is evident that the water extraction removes most (71 %) of the ash of the mould pad. The barium precipitate consisted of about 12 % barium sulphate and about 88 % barium phosphate.

Several peculiar results were noted in making the colour tests on this first water-extract: the development of a deep green colour with the biuret reagents, and a blue colour with phosphotungstic acid-sodium carbonate. On investigation of the reasons for the blue colour test, it was found that Hunter's [1928] colour test for ergothioneine was strongly positive. Attempts to isolate ergothioneine by two different procedures [Tanret, 1909; Williamson and Meldrum, 1932] failed.

The second water-extract (following the removal of the lipins) differed in a few particulars from the first. It gave a negative biuret test, but positive Millon, Hopkins-Cole and xanthoproteic tests. No proteins were present, however, as was shown by the absence of any precipitate with trichloroacetic acid or upon heating.

Extraction with cold 1 % KOH solution removed about 2/5 of the nitrogen originally present in the mould pad (39·7 %, batch 28; 41·2 %, batch 31). The extract gave positive biuret, xanthoproteic, Millon and Hopkins-Cole tests. A heavy white precipitate was formed upon acidification. A considerable amount of carbohydrate was present in the extract as was evidenced by the fact that the acid hydrolysate of the extract strongly reduced Fehling's solution.

Various procedures were used in an attempt to isolate the protein from the extract, including fractional precipitation by alcohol and acetone, saturation with zinc sulphate, precipitation by trichloroacetic and tungstic acids; but none of these methods gave a protein preparation free from carbohydrate. In fact, the highest nitrogen content found in any fraction was only 12·3 %. It may be that the carbohydrate and protein were combined as in the case of glycoproteins. Other investigators have reported similar low nitrogen figures for mould proteins, 12–12·9 % [Thomas and Moran, 1914; Clutterbuck *et al.*, 1932].

The extraction with boiling 4 % KOH removed a quantity of material which was rather low in nitrogen (4·9–5·8 %). The extract was coloured dark brown, and the precipitates obtained from it were also very dark. Part of the alkali used in the extraction was not accounted for on titration with acid, presumably because of the presence of acidic groups in the extracted material.

The nature of the resistant, alkali-insoluble residue has been discussed elsewhere [Norman and Peterson, 1932]. Hydrolysis for 24 hours with 20 % HCl brought 78 % of its nitrogen into solution. Glucosamine hydrochloride readily crystallised out of the acid hydrolysate and accounted for 62 % of the nitrogen in the resistant residue.

Partition of the water-soluble nitrogen.

The ammonia-nitrogen of the water-extract (batch 28) was very low, only 3·9 % of the total. It is quite obvious that the mould, although it may absorb nitrogen in the form of ammonium salts, does not store it in the hyphae for any length of time in that form.

A rough attempt was made to determine the amount of urea present by precipitation with xanthydrol and determination of the nitrogen content of the precipitate. This was found to be 3·9 % of the total nitrogen in the extract.

The amounts of amide-nitrogen found varied within wide limits according to the method used. The lowest figure, 3·5 %, was obtained by hydrolysis with $2N$ H_2SO_4 for 6 hours [Vickery, 1931]. The highest figure, 14·8 %, was found after hydrolysis with 20 % HCl for 24 hours. The difference between these two figures (11·3 %), is formed from unknown compounds during the severe hydrolysis with 20 % HCl. The maximum figure is slightly less, however, than that reported by Vorbrodt [1926] who found 20·9 % amide-nitrogen in the hydrolysate of the water-extract of *A. niger*.

In some batches of mould the first water-extract contained some substance which had the power of catalytically decomposing nitrous acid to nitric oxide with extreme rapidity. Consequently, attempts to determine the α-amino-nitrogen of these extracts by the Van Slyke method were attended with great difficulty. The apparent amino-nitrogen of these extracts varied from 80 to 110 % of the total nitrogen in the extract. However, in the water-extract of a batch that behaved normally the free amino-nitrogen before acid hydrolysis was 37·5 % of the total nitrogen in the extract. (Table I.)

The nitrogen partition of the water-extract from batches 31 and 34 is given in Table II. The basic nitrogen in the acid-hydrolysed extract varied from 14·9 %

Table II. *Nitrogen partition of water-extract.*

	Batch 31 % of total N	Batch 34 % of total N
Ammonia-N	18·7	18·1
Humin-N	6·6	5·4
Phosphotungstic acid precipitate-N	14·9	20·0
Phosphotungstic acid filtrate, amino-N	58·9	58·4
Phosphotungstic acid filtrate, non-amino-N	1·5	0·3
Total recovered	100·6	102·2

of the total (batch 31) to 20·0 % (batch 34). Vorbrodt [1926] found 6·2 % basic nitrogen in the acid hydrolysate of the water-extract of *A. niger*.

Although the water-extract gave no clearly positive test for peptides, it contained a large proportion of peptide-nitrogen. Before hydrolysis, the α-amino-nitrogen was 30·2 % and after hydrolysis 67·7 %, giving a difference of 37·5 % as combined amino-nitrogen.

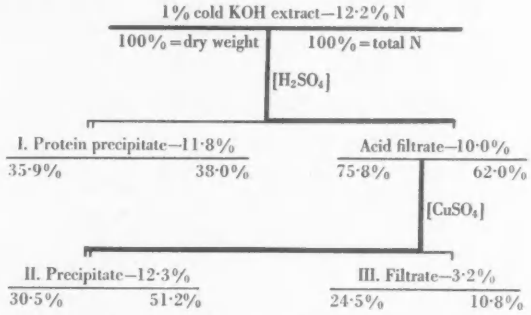
The lipin fraction contained a small amount of nitrogen which was not investigated further.

Fractionation and partition of the alkali-soluble nitrogen.

The cold alkaline extract was found to contain two types of protein—one soluble in acid solution, the other precipitable on acidification. The acid filtrate, however, also contained considerable amounts of carbohydrate, and the acid-soluble protein and carbohydrate had almost identical solubilities, so much so that it was exceedingly difficult to separate the two. For example, tungstic acid, which completely precipitated the protein, was found to precipitate the carbohydrate also.

The procedure finally adopted is shown in the following scheme: precipitation of first protein fraction with acid at p_H 4·8–5·2, precipitation of second protein

fraction with copper sulphate at p_H 6·2–6·5, with most of the carbohydrate remaining in the filtrate.



Fractionation of nitrogen in first alkaline extract (batch 34).

Explanation: Figures above the line and on the right indicate percentage of nitrogen in each fraction (ash-free basis). Figures below the line on the right show the percentage of the total nitrogen which was contained in each fraction. Figures below the line on the left indicate the percentage of the total solids (ash-free) found in each fraction.

The first protein precipitate was a tan-coloured powder; it contained 1·2 % ash, 11·8 % N and 0·4 % P. It was soluble in dilute alkalis, precipitated by acidification with mineral acids (partly dissolved by an excess), acetic, trichloroacetic, phosphotungstic and tungstic acids. It gave all the usual protein colour tests and in addition reduced Fehling's solution after acid hydrolysis.

The copper sulphate precipitate dissolved in excess acid with the formation of a greenish blue colour, and in excess alkali with the formation of a deep purple colour. It contained 12·3 % N and 2·4 % P (ash-free basis), and varying amounts of ash, 12 to 37 %. This preparation was precipitated from acid solution by phosphotungstic and tungstic acids. The acid hydrolysate of this preparation reduced Fehling's solution.

The filtrate from the copper sulphate precipitate no longer gave any protein colour tests but contained 3·2 % N (ash-free basis), of which 36 % was free amino-nitrogen. After acid hydrolysis, it strongly reduced Fehling's solution. The distribution of the nitrogen and of ash-free solids in the three fractions is shown in the scheme.

Table III. *Nitrogen partition of the combined alkaline extracts (batch 31).*

	Extract % of total N	H_2SO_4 ppt. % of total N	$CuSO_4$ ppt. % of total N
Ammonia-N	6·6	4·9	6·7
Humin-N	8·2	7·7	7·9
Phosphotungstic acid precipitate-N	24·4	20·5	31·6
Phosphotungstic acid filtrate, amino-N	51·5	61·5	55·0
Phosphotungstic acid filtrate, non-amino-N	6·1	6·9	0·0
Total recovered	96·8	101·5	101·2

Table III shows the nitrogen partition of the combined alkaline extracts of batch 31, in which 55·5 % of the total N of the mycelium was alkali-soluble. This material differed markedly from the water-extract in that only one-third as much

ammonia was liberated by acid hydrolysis. The alkali-soluble nitrogen also contained a considerably larger amount of basic nitrogen than did the water-extract. The copper sulphate precipitate contained 50 % more basic nitrogen than the acid precipitate, which fact may be due to the presence in the former fraction of most, if not all, of the mould nucleic acid.

This difference between the two fractions was still more marked in the nitrogen partition (Table IV) carried out on the two fractions from the cold 1 % KOH

Table IV. *Nitrogen partition of protein fractions from first alkaline extract (batch 34).*

	H_2SO_4 ppt. % of total N*	$CuSO_4$ ppt. % of total N
Ammonia-N	4·9	8·7
Humin-N	5·8	13·8
Phosphotungstic acid precipitate-N	22·8	38·0
Phosphotungstic acid filtrate, amino-N	60·2	36·5
Phosphotungstic acid filtrate, non-amino-N	3·9	1·8
Total recovered	97·6	98·8

* Average of two determinations.

extract (containing 43·2 % of the total mycelial nitrogen) of batch 34. In the copper sulphate precipitate of this extract 38 % of the nitrogen was basic and only 36·5 % monoamino-nitrogen. This fraction would very likely serve as a convenient source of mould nucleic acid.

Summary.

The solubility and partition of the nitrogenous constituents in the mycelium of *A. fischeri* have been determined. The mould-nitrogen was most easily extracted when fresh, unaltered mould tissue was used. The mycelium contained about 30 % water-soluble non-protein-N, 55 % of alkali-soluble protein-N, and 12 % of alkali-insoluble residue-N.

The alkali-soluble protein consisted chiefly of two fractions, one precipitated by acids, the other acid-soluble but precipitated by copper sulphate.

The acid-insoluble protein contained 1·2 % ash, 11·8 % N and 0·4 % P. Of the N in this protein, 22·8 % was basic N and 60·2 % was monoamino-N.

The acid-soluble protein contained a variable, but generally high, ash (12–37 %), 12·3 % N (ash-free basis), and 2·4 % P (ash-free basis). This protein contained 38·0 % of basic N and only 36·5 % of monoamino-N.

The resistant residue contained 2·3 % N. Of the nitrogen in this residue, 62 % has been isolated as glucosamine-N.

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LXXIV. INTERACTION OF VITAMIN D AND DIETARY FACTORS IN THE HEALING OF RICKETS IN RATS.

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(*Received February 23rd, 1934.*)

Rickets produced in rats by a high calcium-low phosphorus diet may be healed either by the administration of vitamin D or by the addition of a source of phosphorus to the diet. The estimation of the influence of small additions of sources of phosphorus or of other related changes in composition of the diet has, however, received much less attention than the relation between the degree of healing and the dose of vitamin D. It is of considerable practical importance to find some means of expressing the antirachitic effect of such dietary factors, both alone and in conjunction with vitamin D, more especially in those cases where a measure is to be made of the vitamin D content of foodstuffs containing phosphorus compounds. From the theoretical point of view, the discovery of such a relation would throw some light on the synergism of vitamin D and phosphorus compounds.

The exact form of the effect of added phosphorus compounds on the healing produced by vitamin D may be complicated. However, two simple alternative approximations suggest themselves. The antirachitic activity might be additive, *i.e.* equivalent to that of an additional dose of vitamin D, or it might be multiplicative, *i.e.* multiplying the effect of doses of vitamin D actually administered. Key and Morgan [1932] found that by changing the ratio of calcium to phosphorus from 4 : 1 to 2 : 1 in a certain rachitogenic diet, a healing effect was produced equal to that of a dose of 0·7 International Unit (I.U.) of vitamin D with the original diet. Again, Lecoq and Villette [1933, 2] found the curative dose of vitamin D for rachitic rats on the Randoin-Lecoq diet to be 20 I.U. incorporated in 100 g. of diet. The curative doses of various phosphates had previously been found [Lecoq and Villuis, 1932; Lecoq and Villette, 1933, 1], and they now proceeded to assign to these compounds potencies in antirachitic units. This form of expressing the effect of changes in mineral composition of the diet is convenient and is justifiable when vitamin D is not administered simultaneously; but it is unjustifiable to assume, without further experiment, any particular relation of the combined effect to the effects of the two influences separately. A certain ambiguity may arise in this connection, and it is important to make quite clear the distinction between the sum of the effects, measured separately, of two influences and the effect of their sum when applied simultaneously. The expression of both dietary changes and doses of vitamin D in antirachitic units would seem to imply that these two influences should be summed in order to calculate their combined effect. Both groups of workers do, in fact, conclude that the effect of simultaneous administration of phosphate and of vitamin D will be the sum of their separate effects.

When partial healing is produced by administration of vitamin D to rats on a rachitogenic diet it is commonly recognised that although the general form of

the curve relating dose to healing effect must be S-shaped, the central portion is approximately logarithmic. Thus for a considerable range of dose the degree of healing is proportional to the logarithm of the dose. This relation has been found for radiographic measurements of healing by Everse and Van Niekerk [1931] and by Bourdillon *et al.* [1931], for the line test by R. S. Morgan [1932], for growth response by Coward *et al.* [1932], and also for measurements by the prophylactic method using A/R ratios [Hume *et al.*, 1932] or a radiographic scale [Bourdillon and Bruce, 1932]. In the case of the radiographic scale used by Bourdillon *et al.* [1931], this relation is expressed by the equation

$$\log_{10} D_2 - \log_{10} D_1 = \frac{(n_2 - n_1)\log_{10} 2}{2},$$

where n_1 and n_2 are the scale numbers of litter-mates which have received doses D_1 and D_2. With a homogeneous stock of rats kept under uniform conditions, the effect of animal variation is reduced to a minimum, and the mean degree of healing, n, in a group of rats will be fairly closely proportional to the logarithm of the dose, log D. The graphical relation between n and log D in an ideal case will be a straight line, *e.g.* (*a*) in Fig. 1.

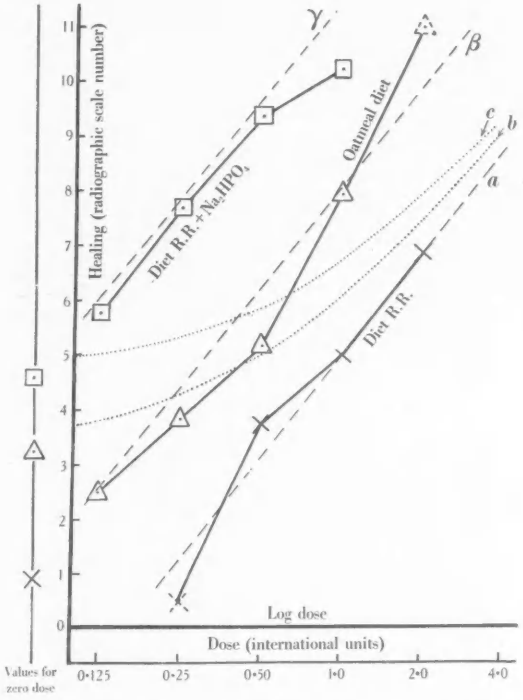

Fig. 1. Comparison of experimental and theoretical relations between log dose and healing.

——— Lines joining experimental points.
— — — Ideal line for diet R.R. (*a*), and lines calculated from this on multiplicative basis (β and γ).
..... Curves calculated on additive basis (*b* and *c*).

If, now, an alteration in the diet is equivalent to the addition of a dose of vitamin D, say d, the relation becomes

$$n \propto \log (D+d),$$

and the relation between the logarithm of the actual dose given and the scale number is no longer linear, *cf.* curves (*b*) and (*c*) in Fig. 1, which deviate considerably from the original line at low values of D, but approach it at high values.

If, however, an alteration in the diet has the effect of multiplying the dose by a factor ϕ, the relation becomes

$$n \propto \log \phi D = \log \phi + \log D.$$

The relation is still linear, but the line is shifted a distance along the n axis corresponding to $\log \phi$, and remains parallel with the line for the original diet, as is shown in Fig. 1, curves β and γ. At higher doses there is a clear and increasing difference between the values given by additive and multiplicative constants, corresponding to the increasing difference between the sum of these two constants and their product. The curves *b* and *c* in Fig. 1 show that a constant addition to the actual dose given has a varying effect with different doses, whilst the lines β and γ in Fig. 1 show that multiplication of the dose by a constant factor produces a constant addition to the effect, increasing the healing by the same number of degrees on the scale with any dose.

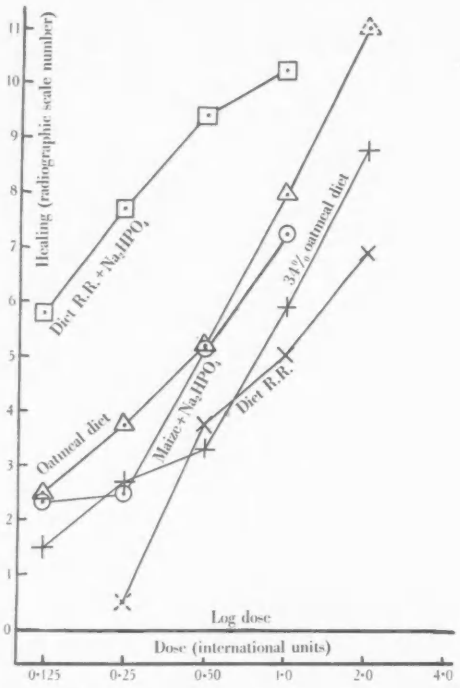

Fig. 2. Relation between log dose and healing. Experimental data for five diets.

In the course of the investigation described in the next paper [Bruce and Callow, 1934] we had occasion to compare quantitatively the calcifying powers of diets containing either different cereals or different forms of phosphorus, and we had to settle the question of how these differences were to be expressed. In Fig. 2 is shown the result of plotting degree of healing against logarithm of the dose with five diets for which suitable data are available. The picture corresponds reasonably well with a series of parallel lines, and there is no resemblance whatever to a series of convergent curves such as would be required by additive influences. Confirmation is afforded by Fig. 1, in which data obtained for the two least rachitogenic diets (oatmeal and R.R. + Na_2HPO_4) are plotted and compared with curves calculated on the basis of additive constants (b and c) or multiplicative factors (β and γ) applied to the standard rachitogenic diet (R.R.). The evidence is decisively in favour of multiplicative factors for both diets.

Experimental.

Data.

Full details of the technique and of the diets used are given in the next paper [Bruce and Callow, 1934]. The data from which the graphs in Figs. 1 and 2 have been constructed are given in Table I.

Table I.

Description of diet	Dose (International Units)	Healing Mean radiographic scale no.	No. of rats
R.R. (routine rachitogenic diet: 20% maize, 48% white flour, 32% basal mixture)	Nil	0·9	4
	(0·25	0·5	2)
	0·5	3·76	15
	1·0	5·02	45
	2·0	6·9	5
34% oatmeal (a mixture of equal parts of R.R. (above) and oatmeal diet (*cf.* below))	Nil	0·33	3
	0·125	1·5	3
	0·25	2·7	3
	0·5	3·3	6
	1·0	5·9	5
	2·0	8·75	4
Maize + Na_2HPO_4 (68% maize, 32% basal mixture, 2·46 g. Na_2HPO_4 (anhydr.) per kg.)	Nil	0·5	4
	0·125	2·33	3
	0·25	2·5	7
	0·5	5·14	18
	1·0	7·22	11
Oatmeal (68% oatmeal, 32% basal mixture)	Nil	3·23	58
	0·125	2·5	4
	0·25	3·85	17
	0·5	5·19	50
	1·0	7·95	17
	(2·0	11·0	2)
R.R. + Na_2HPO_4 (R.R. (above) + 8 g. Na_2HPO_4 (anhydr.) per kg.)	Nil	4·57	31
	0·125	5·79	19
	0·25	7·7	41
	0·5	9·38	13
	1·0	10·25	4

Calculation of additive constants.

A straight line is drawn through the points for diet R.R. in Fig. 1. The doses, as given by this line, corresponding to the scale numbers given by rats on oatmeal diet and on R.R. + Na_2HPO_4 diet with no administered dose, determine the values of d for these diets. The values are $d = 0{\cdot}51$ unit for the oatmeal diet, and

$d=0{\cdot}84$ unit for the R.R.+Na_2HPO_4 diet. The theoretical curves (*b*) and (*c*) are then plotted, substituting, at values of the logarithm of the actual dose, values for the scale number corresponding to an assumed dose, $(D+d)$.

Calculation of multiplicative constants.

The values of the multiplicative factor are derived from a series of comparisons of litter mates on diet R.R. and the other two diets, which is described in detail in the paper following this. The relation for the radiographic scale, *viz.*

$$\log_{10} D_2 - \log_{10} D_1 = \frac{(n_2 - n_1)\log_{10} 2}{2}$$

becomes $$D_2/D_1 = \text{antilog}\,[(n_2 - n_1) \times 0{\cdot}1505].$$

In the case of two diets A and B, the value of D_2/D_1 which is found is an "apparent dose ratio," a_A/a_B. If the ratio of the doses actually administered is d_A/d_B, then the apparent dose ratio is given by

$$\frac{a_A}{a_B} = {}_A\phi_B \times \frac{d_A}{d_B},$$

where ${}_A\phi_B$ is the multiplicative factor for diet A with respect to diet B, and

$${}_A\phi_B = \frac{a_A}{a_B} \times \frac{d_B}{d_A}.$$

Referred to diet R.R., the value for ϕ for the oatmeal diet, determined from the mean of a number of direct comparisons, is 3·2, whilst for the R.R.+Na_2HPO_4 diet, $\phi = 8{\cdot}35$.

Summary and Conclusions.

When vitamin D is administered to rachitic rats on a diet containing excess of calcium, the influence of increasing or altering the source of phosphorus may be expressed as a factor multiplying the dose of vitamin D. Expression of the dietary influence as the addition of a certain number of antirachitic units to the actual dose of vitamin D gives figures which are not concordant with experimental results. The combined antirachitic effect of changes in the mineral composition of a diet and of simultaneous administration of vitamin D is thus greater than the effect of the sum of the two acting separately. It is actually more nearly proportional to the product of two factors representing these antirachitic influences.

It is a pleasure to record our thanks to Dr R. B. Bourdillon for his helpful criticism in the course of preparing this work for publication.

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LXXV. CEREALS AND RICKETS. THE RÔLE OF INOSITOLHEXAPHOSPHORIC ACID.

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As the result of a long series of investigations, E. Mellanby [1921; 1922; 1924; 1925; 1926; 1930; 1931; *cf.* Green and Mellanby, 1928] and M. Mellanby [1929] have demonstrated that certain cereals contain an anticalcifying factor having the effect of producing or intensifying rickets in dogs or rats and of retarding calcification of the teeth in dogs. Oatmeal, for instance, was found to have a marked anticalcifying effect in comparison with white flour or rice when the diet contained a large proportion of cereal. M. Mellanby [1929] summarised the evidence as to the nature of this factor as follows: "It has been impossible to obtain evidence to show that the anticalcifying action of cereals on the teeth is due to any known constituent. Neither the fat, carbohydrate, calcium and phosphorus (absolute and relative amounts) nor the acid-base ratio of the mineral constituent explains this action of cereals. There is some evidence that it is associated with protein and liberated from it by proteolytic digestion."

Certain points with regard to these investigations should be noted. The great majority of the experiments were carried out with dogs, which, unlike rats, have a low natural ability to make use of dietary calcium and phosphorus in the absence of vitamin D. It is possible, however, to assume that, with a low level of vitamin D, the Ca/P ratio will have a similar effect in controlling absorption of these elements in dogs and rats. Further, since the effect of adding calcium carbonate was to improve calcification, though only slightly, it seems justifiable to regard the diets as being deficient in calcium. The Ca/P ratio of the cereal has however been over-emphasised in relation to that of the diet as a whole. The cereals used all have calcium contents which are small in comparison with those of the other constituents of the diet, but the phosphorus contents are considerable. Using the figures given by E. and M. Mellanby, it will be found that a diet containing oatmeal (P, 0·392 %) will have a lower Ca/P ratio than a diet containing rice (P, 0·096 %), although the cereals alone have Ca/P ratios of 1 : 5·7 and 1 : 10·7, respectively.

Steenbock *et al.* [1930] compared the calcifying powers of diets containing different cereals with supplements of calcium carbonate and phosphoric acid, using the percentages of ash in the femurs of rats as the basis of comparison. With 1, 2 or 3 % of calcium carbonate in the diet, the calcifying power was in the order: whole wheat > rolled oats > yellow maize, which was the same order as that of the phosphorus contents. Without calcium carbonate, whole wheat showed slightly less calcifying power than the other two cereals. When the phosphorus content was equalised by addition of phosphoric acid, the order of calcifying power of the diets was practically reversed, although the differences were smaller. They say, "Equalisation of the phosphorus content of the cereal rations did not make them equally effective in bone formation, but it did make

the corn (maize) more effective with its $CaCO_3$ supplement. It must however be considered that inorganic phosphorus added as phosphoric acid may not be equivalent in physiological properties to the organic phosphorus compounds in the cereals. Our experiments certainly demonstrate the fact that inequality of phosphorus intake is not the sole reason for the difference in calcifying action of the various cereals."

Fine [1930] also confirmed the existence of differences in the calcifying properties of cereals, but was inclined to ascribe such differences to variations in vitamin D content rather than to variations in content of an anticalcifying factor.

Quite recently György *et al.* [1933] concluded that the existence of a rachitogenic factor in cereals, although lacking material proof, was supported strongly by their results, although they differed from Mellanby in finding that maize meal was most highly rachitogenic. They also confirmed the reduction of the rachitogenic effect by treatment with acid, but showed that the high proportion of sodium chloride resulting from subsequent neutralisation was alone capable of exerting a slight antirachitic effect.

It seemed to us that the influence of absolute and relative amounts of calcium and phosphorus on calcification was worthy of further study. Although there were no grounds for supposing that the elusive anticalcifying factor was entirely a matter of calcium and phosphorus content, a quantitative analysis of the cereal effect from that point of view was a necessary preliminary to further investigation. The suggestion of Steenbock *et al.* that the phosphorus compounds of cereals differed in their biological availability, might, moreover, contain an important clue to the solution of the problem, and attention should be paid to the nature of the phosphorus compounds.

The fact that in the work of Steenbock *et al.* the cereal with the highest phosphorus content had the lowest calcifying power when the phosphorus contents of the diets had been equalised strongly suggests that cereals contain a form of phosphorus less available than phosphoric acid. Independent evidence does, in fact, exist that this is the case.

The mineral components of a diet are too frequently expressed as a total percentage only, with little or no reference to the actual form in which those elements are present. Most cereals are relatively rich in phosphorus, but from 50–80 % of this is present in the form of inositolphosphoric acids, "phytin-phosphorus" [for literature, *cf.* Koehler, 1926], and it is a debatable question whether phosphorus in this form is absorbed at all from the digestive tract. The earlier literature is reviewed by Schaumann [1910]. Starkenstein [1910] concluded from his own investigations on organic phosphates in human urine that only a small proportion of orally administered inositolhexaphosphate was absorbed unchanged, the great part being hydrolysed by bacteria in the intestine. Plimmer [1913, 1] demonstrated that inositolhexaphosphoric acid diffused only slowly through parchment membranes, and that the acid was not hydrolysed by intestinal extracts from carnivora or herbivora. He concluded that there was no evidence of direct absorption, but that after hydrolysis by an enzyme in the foodstuff or by bacterial action it would enter the circulation as inositol and phosphoric acid.

As regards the availability of phytin-phosphorus in rickets, the evidence in the literature is conflicting. Eddy *et al.* [1922] demonstrated that supplements of phytin were ineffective in protecting rats from rickets when added to Sherman-Pappenheimer's diet No. 84. More recently there has been a controversy about the antirachitic activity of "Vitaphos," a preparation of calcium magnesium

inositolhexaphosphate. Von Wendt [1930] claimed that "Vitaphos" contained a form of vitamin D which was not toxic in excessive doses. Vonder Mühll [1931] contested this and demonstrated that the curative effect on rickets in rats could be explained as a result of the addition of phosphorus compounds. A more detailed investigation was recorded by Bleyer and Fischler [1931, 1, 2], who claimed that "Vitaphos" restored the composition of rats' bones to normal when added to McCollum's diet No. 3143 in the proportion of from 3 to 7 %. Their experiments are however open to several objections.

In the present communication there is described an investigation of the differences in calcifying power between cereals and of the availability of phytin-phosphorus, using the method of measuring the calcifying powers of diets which has been described in the preceding paper.

The series of diets compared were all of the high calcium-low phosphorus type, made up from 32 parts of a basal mixture and 68 parts of cereal. In certain cases a source of phosphorus was added. All contained 2 % of calcium. One diet, the "routine rachitogenic diet," had a mean Ca/P ratio of 10·3/1; the others had a mean Ca/P ratio of 5·5/1. In this way a comparison has been made of the effects due to the addition to rachitogenic diets of phosphorus in various forms,

(*a*) as a component of the cereal itself,

(*b*) as disodium hydrogen phosphate, and

(*c*) as sodium or calcium magnesium inositolhexaphosphates.

Further, the effects on oatmeal of cooking and of treatment with dilute acid have been investigated.

It has not been found possible to apply the same method to diets of the low calcium-high phosphorus type, but we have included here an account of some preliminary qualitative comparisons of such diets.

Experimental.

Analytical methods.

The determinations of calcium and phosphorus in dietary constituents and in diets were made by Neumann's method as described by Aron [1912]. Air-dry samples were taken, as variation in the content of moisture affects the absolute figures only slightly, without altering the relative proportions of calcium and phosphorus found.

Phytin-phosphorus (as inositolhexaphosphoric acid) was determined by the method of Heubner and Stadler [1914], with certain of the modifications introduced by Andrews and Bailey [1932].

Diets and materials. The "basal" portion of the diets was composed of:

Wheat gluten	20
Meat powder	6
Calcium carbonate	5
Sodium chloride	1
	32

The components were drawn from the same stocks throughout the investigation. In the "routine rachitogenic diet" (R.R.), and in some of the other experimental diets, the "cereal" portion consisted of a mixture of yellow maize meal (20 parts) and white flour (48 parts); in other diets it consisted of oatmeal or of maize.

The following values were found for certain of the constituents.

Meat powder, Ca, 0·045%; P, 0·75%. Wheat gluten, P, 0·148%. Maize meal, Ca, 0·04%; P, 0·37% (P soluble in HCl, 0·35%; phytin-P, 0·34%; phytin, as $C_6H_{18}O_{24}P_6$, 1·2%.) White flour,

Ca, 0·018%; P, 0·078% (P soluble in HCl, 0·026%; phytin-P, 0·014%; phytin, as $C_6H_{18}O_{24}P_6$, 0·05%). The oatmeal was "Ingles Midlothian Scotch, fine," and contained Ca, 0·06%; P, 0·406% (P soluble in HCl, 0·35%; phytin-P, 0·32%; phytin, as $C_6H_{18}O_{24}P_6$, 1·1%). Disodium hydrogen phosphate was used as the anhydrous salt, dried at 120° and ground to pass 80 mesh. The calcium magnesium inositolhexaphosphate used was a commercial brand of "phytin." Found: Loss at 100°/15 mm., 13·2%, and, referred to anhydrous material, Ca, 11·8%; P, 22·0% (phytin-P, 22·0%; phytin, as $C_6H_{18}O_{24}P_6$, 78·1%). The sodium salt was prepared from this by the method of Posternak [1921], recrystallised from water, dried partially *in vacuo* and ground to pass 80 mesh. (Found: P, 19·2%; phytin-P, 18·5%; inorganic P, 0·7%.) The mixing of small amounts of these addenda with the diets was carried out in batches of 2–4 kg. in a large ball mill for a period of 2·5–3 hours.

We have met with some difficulty in reproducing the exact composition of a diet as regards the total calcium and phosphorus content when the diets have been mixed in bulk. For this reason, where it was necessary to make up more than one batch of any given diet, each batch has been analysed separately. Thus the figures given represent the actual diets which were employed in feeding.

Biological methods.

The practical details of the rat tests were essentially as described by Bourdillon *et al.* [1931]. Rats weaned at approximately 4 weeks of age (50 g. weight) were put on the R.R. diet for 2 weeks, and then the knee-joints were radiographed under anaesthesia to confirm the satisfactory development of rickets. The rats in each litter were then sorted into pairs comparable in weight and sex, as far as was possible, and transferred to the diets to be tested, the two members of a pair being put on different diets. Daily doses of a standardised solution of irradiated ergosterol in olive oil were given in most cases. In general it was arranged that the two members of a pair should have either equal doses or doses which, taking into consideration the differences between the two diets, would produce comparable degrees of healing. 12 doses were given in 14 days. At the end of 14 days of this regime, the rats were killed by coal-gas and the knee-joints again radiographed. The degree of healing in each case was then estimated by comparison with the standard scale.

The general theory of the method used, and its relationship to the method of estimation of vitamin D by radiography has been discussed in the preceding paper [Bruce and Callow, 1934]. For a pair of rats on different diets, A and B, showing healing scale numbers n_2 and n_1, the apparent dose ratio is given by:

$$\frac{a_A}{a_B} = \text{antilog}\,[(n_2 - n_1) \times 0{\cdot}1505].$$

If ${}_A\phi_B$ is the multiplicative factor for diet A with respect to diet B, *i.e.* the influence of diet A in multiplying the apparent magnitude of a dose of vitamin D, the effect of which has been determined for diet B, and if the ratio of the doses actually administered is d_A/d_B, then

$$\frac{a_A}{a_B} = {}_A\phi_B \times \frac{d_A}{d_B},$$

and

$${}_A\phi_B = \frac{a_A}{a_B} \times \frac{d_B}{d_A}.$$

Comparison of high calcium-low phosphorus diets.

In the following section the protocols are given in full in one case, in order to illustrate the method of calculation, but in the other cases only in summary form.

(i) *Comparison of maize + white flour mixture with oatmeal.* Diet R.R. (Ca, 1·97%; P, 0·227%; Ca/P = 8·7) was compared with diet No. 10 (Ca, 2·0%; P, 0·406%; Ca/P = 4·9), in which the cereal portion consisted entirely of oatmeal.

(*a*) In the first experiment, with two litters divided into 8 pairs, the doses of vitamin D in International Units, and the degrees of healing obtained were as given in Table I.

Table I.

	Diet No. 10		Diet R.R.		
Litter No.	Dose (International Units)	Healing. (Scale Units)	Dose. (International Units)	Healing. (Scale Units)	Difference in healing. (No. 10 – R.R.) Δ
384	Nil	2	Nil	1	1
	0·5	4·5	0·5	1·5	3
	1	10	1	5	5
	2	11	2	4	7
1776	Nil	1	Nil	0	1
	0·5	9	0·5	4·5	4·5
	1	10·5	1	7	3·5
	2	11	2	6	5

The mean difference in degrees of healing between rats receiving equal doses of vitamin D on diets No. 10 and R.R. was 3·75, and, from this:

Apparent dose ratio $a_{10}/a_{\text{R.R.}}$ = antilog ($\Delta \times 0{\cdot}1505$) = 3·7.

Since the actual dose ratio, $d_{10}/d_{\text{R.R.}}$, is unity, this apparent dose ratio is equal to ${}_{10}\phi_{\text{R.R.}}$, the ratio of the antirachitic potencies of diets No. 10 and R.R.

(*b*) In the second experiment (Table II) two litters were divided into 5 pairs in which the dose ratio $d_{10}/d_{\text{R.R.}}$ was 1 : 4.

Table II.

	Diet No. 10		Diet R.R.		
Litter No.	Dose. (International Units)	Healing. (Scale Units)	Dose. (International Units)	Healing. (Scale Units)	Difference in healing. (No. 10 – R.R.) Δ
405	0·125	2	0·5	3	–1
	0·25	5	1	6	–1
	0·5	5·5	2	6·5	–1
1822	0·125	1	0·5	4·5	–3·5
	0·25	5	1	6	–1

Mean value of $\Delta = -1{\cdot}5$.

$$ {}_{10}\phi_{\text{R.R.}} = \frac{d_{\text{R.R.}}}{d_{10}} \times \frac{a_{10}}{a_{\text{R.R.}}} = \frac{4}{1} \times \text{antilog}\,(-1{\cdot}5 \times 0{\cdot}1505) = 2{\cdot}4. $$

The mean value of ${}_{10}\phi_{\text{R.R.}}$ is 3·2.

(ii) *Addition of calcium magnesium inositolhexaphosphate to maize + white flour mixture.* (*a*) Diet R.R. (Ca, 1·98%; P, 0·195%; Ca/P = 11·5): 8 rats, dose 1 unit; 2 rats, dose 0·5 unit.

Diet No. 18 was made up with the same cereal and base, except that the calcium carbonate was reduced from 5% to 4·77%, and calcium magnesium inositolhexaphosphate was added in the proportion of 27·6 g. to 2993 g. of diet. (Ca, 1·93%; P, 0·364%; Ca/P = 5·3): 10 rats, dose 0·5 unit.

8 pairs, $d_A/d_B = 0{\cdot}5$, mean $\Delta = -1{\cdot}75$, ${}_{18}\phi_{\text{R.R.}} = 1{\cdot}09$.

2 pairs, $d_A/d_B = 1$, mean $\Delta = +0{\cdot}75$, ${}_{18}\phi_{\text{R.R.}} = 1{\cdot}30$.

${}_{18}\phi_{\text{R.R.}}$ (mean, 10 pairs) = 1·13.

(*b*) Diet R.R. (Ca, 1·90; P, 0·203%; Ca/P = 9·4): 12 rats, dose 1 unit.

Diet No. 24 (equivalent to No. 18) (Ca, 1·94%; P, 0·348%; Ca/P = 5·6): 10 rats, dose, 1 unit.

10 pairs, $d_A/d_B = 1$, mean $\Delta = 0{\cdot}81$, ${}_{24}\phi_{\text{R.R.}} = 1{\cdot}32$.

For 20 pairs the mean factor for the phytin diet is 1·23.

(iii) *Addition of disodium hydrogen phosphate to maize + white flour mixture.* (*a*) Diet R.R. (as above): 2 rats, dose 0·5 unit; 2 rats, dose 1 unit; 1 rat, dose 2 units.

Diet No. 15, consisting of diet R.R. with the addition of 8·11 g. of Na_2HPO_4 anhydr. per kg. (Ca, 1·90%; P, 0·392%; Ca/P = 4·9): 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 1 rat, dose 0·5 unit.

5 pairs, $d_A/d_B = 0·25$, mean $\Delta = 0·80$, ${}_{15}\phi_{R.R.} = 5·27$.

(*b*) Diet R.R. (Ca, 1·98%; P, 0·195%; Ca/P = 10·2): 7 rats, dose 1 unit.

Diet No. 19 (as No. 15, above) (Ca, 1·98%; P, 0·368%; Ca/P = 5·4): 7 rats, dose 0·25 unit.

7 pairs, $d_A/d_B = 0·25$, mean $\Delta = 2·36$, ${}_{19}\phi_{R.R.} = 9·06$.

(*c*) Diet R.R. (Ca, 1·74%; P, 0·186%; Ca/P = 9·4): 6 rats, dose 1 unit.

Diet No. 21 (as Nos. 15 and 19, above) (Ca, 1·91%; P, 0·359%; Ca/P = 5·3): 6 rats, dose 0·25 unit.

6 pairs, $d_A/d_B = 0·25$, mean $\Delta = 2·67$, ${}_{21}\phi_{R.R.} = 10·08$.

Mean, for all groups, 18 pairs, ${}_{PO_4}\phi_{R.R.} = 8·35$.

(iv) *Comparison of calcium magnesium inositolhexaphosphate with disodium hydrogen phosphate.* Diet No. 18, containing added calcium magnesium inositolhexaphosphate, *cf.* (ii, *a*), above: 12 rats, dose 0·5 unit.

Diet No. 19, containing added Na_2HPO_4, *cf.* (iii, *b*), above: 12 rats, dose 0·25 unit.

12 pairs, $d_A/d_B = 2$, mean $\Delta = 3·5$, ${}_{18}\phi_{19} = 0·15$.

(v) *Comparison of sodium inositolhexaphosphate with disodium hydrogen phosphate.* Diet No. 48 consisting of diet R.R. with the addition of 14 g. of dry sodium inositolhexaphosphate per kg. (Ca, 2·06%; P, 0·420%; Ca/P = 4·9): 4 rats, dose nil; 6 rats, dose 0·25 unit; 6 rats, dose 0·5 unit.

Diet No. 49, consisting of diet R.R. with the addition of 11 g. of anhydrous Na_2HPO_4 per kg. (Ca, 2·12%; P, 0·418%; Ca/P = 5·1): 4 rats, dose nil; 6 rats, dose 0·125 unit; 6 rats, dose 0·25 unit.

4 pairs, dose nil, mean $\Delta = -4·25$, ${}_{48}\phi_{49} = 0·23$.

12 pairs, $d_A/d_B = 2$, mean $\Delta = -2·29$, ${}_{48}\phi_{49} = 0·23$.

Mean, for 16 pairs, ${}_{48}\phi_{49} = 0·23$.

(vi) *Comparison of oatmeal with maize + white flour mixture containing added disodium hydrogen phosphate.* (*a*) Diet No. 13, consisting of diet R.R. with the addition of 8·66 g. of anhydrous Na_2HPO_4 per kg. (Ca, 2·00%; P, 0·433%; Ca/P = 4·6): 6 rats, dose nil.

Diet No. 10, containing oatmeal, *cf.* (i, *a*), above: 6 rats, dose nil.

6 pairs, dose nil, mean $\Delta = 2·92$, ${}_{13}\phi_{10} = 2·75$.

(*b*) Diet No. 15, *cf.* (iii, *a*): 2 rats, dose nil; 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 2 rats, dose 0·5 unit.

Diet No. 10: 2 rats, dose nil; 2 rats, dose 0·125 unit; 2 rats, dose 0·25 unit; 2 rats, dose 0·5 unit.

8 pairs, $d_A/d_B = 1$, mean $\Delta = 2·5$, ${}_{15}\phi_{10} = 2·38$.

(*c*) Diet No. 21, *cf.* (iii, *c*): 4 rats, dose 0·25 unit; 6 rats, dose 0·5 unit; 4 rats, dose 1 unit.

Diet No. 20 (as No. 10) (Ca, 1·97%; P, 0·377%; Ca/P = 5·2): 4 rats, dose 0·25 unit; 6 rats, dose 0·5 unit; 4 rats, dose 1 unit.

14 pairs, $d_A/d_B = 1$, mean $\Delta = 3·89$, ${}_{21}\phi_{20} = 3·85$.

Mean, for all groups, 28 pairs, ${}_{PO_4}\phi_{OATMEAL} = 3·2$.

(*d*) A repetition of (*c*), but with each diet cooked by stirring into four parts of boiling water and heating for half an hour.

Diet No. 21 *c* (phosphate): 4 rats, dose 0·125 unit; 4 rats, dose 0·25 unit; 4 rats, dose 0·5 unit.

Diet No. 20 *c* (oatmeal): 4 rats, dose 0·25 unit; 4 rats, dose 0·5 unit; 4 rats, dose 1 unit.

12 pairs $d_A/d_B = 0·5$, mean $\Delta = 2·04$, ${}_{21c}\phi_{20c} = 4·06$.

(vii) *Comparison of oatmeal with maize + white flour mixture containing added sodium inositolhexaphosphate.* Diet No. 28, consisting of diet R.R. with the addition of 8·5 g. of dry sodium inositolhexaphosphate per kg. (Ca, 1·90%, P, 0·362%; Ca/P = 5·2): 15 rats, dose 0·5 unit.

Diet No. 20, containing oatmeal, *cf.* (vi, *c*), above: 15 rats, dose 0·5 unit.

15 pairs, $d_A/d_B = 1$, mean $\Delta = -0·50$, ${}_{28}\phi_{20} = 0·84$.

(viii) *Comparison of oatmeal with maize.* Diet No. 37, with maize as sole cereal (Ca, 2·00%; P, 0·353%; Ca/P = 5·7) : 6 rats, dose 0·5 unit.

Diet No. 38, with oatmeal as sole cereal (Ca, 2·07%; P, 0·379%; Ca/P = 5·5): 6 rats, dose 0·5 unit.

6 pairs, $d_A/d_B = 1$, mean $\Delta = -2{\cdot}83$, ${}_{37}\phi_{38} = 0{\cdot}38$.

(ix) *Comparison of oatmeal with maize and added disodium hydrogen phosphate.* Diet No. 39, with maize as the sole cereal and 2·46 g. of anhydrous Na_2HPO_4 added per kg. (Ca, 2·16%; P, 0·365%; Ca/P = 5·9) : 13 rats, dose 0·5 unit; 7 rats, dose 1 unit.

Diet No. 40, with oatmeal as sole cereal (Ca, 2·11%; P, 0·365%; Ca/P = 5·8) : 13 rats, dose 0·5 unit; 7 rats, dose 1 unit.

20 pairs, $d_A/d_B = 1$, mean $\Delta = 0{\cdot}20$, ${}_{39}\phi_{40} = 1{\cdot}07$.

(x) *Comparison of cooked, acid-treated and raw oatmeal.* Diet No. 42 was prepared by boiling 4080 g. of oatmeal for four hours with a mixture of 23 litres of water and 1 litre of concentrated hydrochloric acid. After 1·5 hours the product no longer gave a blue colour with iodine. It was then neutralised with anhydrous sodium carbonate (548 g.), the basal mixture (1920 g.) was stirred in, and cooking continued for a further hour. The moisture content of the product was 66%, and analysis, calculated on the oven-dried diet, gave: Ca, 2·25%; P, 0·364%, Ca/P = 6·2; phytin, as $C_6H_{18}O_{24}P_6$, about 0·007%.

Diet No. 41 was prepared by boiling 4080 g. of oatmeal for 4 hours with 24 litres of water then stirring in the basal mixture and an additional 390 g. of NaCl, and cooking for a further hour. The moisture content of the product was 70%, and analysis, calculated on the oven-dried diet gave: Ca, 2·19%; P, 0·364%; Ca/P = 6·0; phytin, as $C_6H_{18}O_{24}P_6$, 0·64%.

Diet No. 43 was the usual dry mixture, with oatmeal as the sole cereal, but with an additional 56·7 g. of NaCl per kg. (Ca, 1·96%; P, 0·324%; Ca/P = 6·1; phytin, as $C_6H_{18}O_{24}P_6$, 0·66%).

A simultaneous comparison was made on triads of litter-mates, without doses, with 21 rats on diet No. 41, 21 rats on diet No. 42, and 20 rats on diet No. 43.

Diets Nos. 41 and 42, mean $\Delta = 3{\cdot}46$, ${}_{42}\phi_{41} = 3{\cdot}66$.
Diets Nos. 42 and 43, mean $\Delta = 3{\cdot}32$, ${}_{42}\phi_{43} = 3{\cdot}32$.
Diets Nos. 41 and 43, mean $\Delta = 0{\cdot}28$, ${}_{41}\phi_{43} = 0{\cdot}91$.

Comparison of low calcium-high phosphorus diets.

The results obtained from the feeding of low calcium-high phosphorus diets have not been very satisfactory. It was first attempted to produce rickets, suitable for radiographic comparison, either by diets which consisted essentially of those described above, but with the omission of calcium carbonate and with added phosphorus compounds. Only a mild type of rickets was obtained, and the radiographic pictures more nearly resembled those found in prophylactic tests. It was found difficult to secure satisfactory growth on low calcium diets, and a slight tendency towards spontaneous healing was often found from the third week onwards. Three classes of diet were employed, one containing oatmeal and the other two containing maize + white flour mixture with the addition of sodium inositolhexaphosphate or sodium phosphate to bring the total phosphorus content up to the same level. Such slight differences as were observed in the rickets resulting after 14–28 days on these diets indicated that the rachitogenic power of the oatmeal diet was slightly less than that of the other two diets, but differences were so small and variable that no real significance could be attached to the result. Using the type of diet described by Green and Mellanby [1928] we were again only able to produce a mild type of rickets in spite of improved growth, but could not detect a difference between the effect of oatmeal and of maize-white flour mixture.

In another experiment the A/R ratio was used as a measure of calcification. Rats weaned at about 4 weeks old were fed one of three diets containing in common the basal mixture: wheat gluten, 20 parts; meat powder, 6 parts; dried liver, 3 parts; sodium chloride, 1 part; No. 44 contained oatmeal (70 parts),

No. 45 contained white flour (70 parts), and sodium inositolhexaphosphate (1·3 parts), and No. 46 contained white flour (70 parts) and disodium hydrogen phosphate (1·1 parts). The phosphorus contents were practically equal, *viz.* 0·38, 0·36 and 0·38 %, respectively. Rats on each of the diets were killed at intervals of a week, from 4 to 8 weeks after the beginning of the experiment. In Table III are shown the time interval, number of rats killed, and average A/R value.

Table III. *Values of* A/R (*no. of rats in parentheses*).

Time (weeks)	4	5	6	7	8
Diet No. 44 (oatmeal)	0·374 (1)	0·433 (2)	0·374 (2)	0·347 (2)	0·391 (2)
Diet No. 45 (inositol-hexaphosphate)	0·545 (1)	0·384 (2)	0·361 (2)	0·428 (2)	0·495 (2)
Diet No. 46 (phosphate)	0·472 (1)	0·468 (2)	0·458 (2)	0·545 (3)	0·502 (1)

It will be seen that the rats on the phosphate diet, after the fourth week, have a consistently higher value of A/R than the rats on the inositolhexaphosphate diet. The rats on the oatmeal diet have a value of A/R which is generally below the value for the rats on the other diets; this is largely to be accounted for by the fact that the rate of growth was very much higher for these rats than for those on either the phosphate or inositolhexaphosphate diets, and an assessment of the anticalcifying power of the oatmeal diet relative to the other two is not possible. In the two latter cases the rates of growth fell off after the third week. With all three diets the growth was unsatisfactory after the sixth week, and an increase in the A/R ratio occurred.

Discussion.

Results with high calcium diets.

The results of the direct comparisons of high calcium diets (i) with diet R.R., (ii) with diet R.R. containing added phosphate and (iii) with the oatmeal diet are summarised in Table IV, in which the diets in the first column are arranged in order of decreasing antirachitic potency.

Table IV.

Characteristic of Diet A	Ca/P (mean)	$A\phi_B$ Diet B; R.R.	$A\phi_B$ Diet B; R.R. + Na_2HPO_4	$A\phi_B$ Diet B; oatmeal
Acid-treated oatmeal	6·2	—	—	3·3
R.R. + Na_2HPO_4	5·2	8·35	(1)	3·2
Maize + Na_2HPO_4	5·4	—	—	1·1
Oatmeal	5·4	3·2	0·32	(1)
Cooked oatmeal	5·9	—	—	0·91
R.R. + Na inositolhexaphosphate	5·1	—	0·23	0·84
Maize	5·7	—	—	0·38
R.R. + Ca Mg inositolhexaphosphate	5·4	1·2	0·15	—
R.R.	10·3	(1)	0·16	0·32

Considering first the diets which have been directly compared with diet R.R., it is seen that the addition of calcium magnesium inositolhexaphosphate causes quite a small increase in the antirachitic potency of the diet, whilst addition of disodium hydrogen phosphate to give the same content of phosphorus causes a very considerable increase. The oatmeal diet, again with the same total phosphorus content, is in an intermediate position. The comparisons with diet R.R. containing added phosphate confirm these relationships, but the diet with added calcium magnesium inositolhexaphosphate is here slightly less antirachitic than diet R.R., and it is evident that the effect of the addition in these amounts is negligible. Sodium inositolhexaphosphate, on the other hand,

appears to cause a definite increase in antirachitic potency, but it should be noted that the specimen used contained a certain amount of inorganic phosphate.

The oatmeal diet has been compared with five other diets containing the same proportion of cereal and of practically the same composition with respect to calcium and phosphorus. The value of ϕ for acid-treated oatmeal diet is 3·3 when compared with the untreated oatmeal diet. This might equally well be explained either by the assumption that the treatment has rendered the phosphorus more readily available, or by the assumption that treatment with acid has destroyed a rachitogenic factor of some other nature. In support of the first assumption are the facts that inositolphosphates were hydrolysed to phosphates by this treatment and that the availability of the phosphorus was consequently greatly increased. Moreover, the addition of phosphate to diet R.R. yields a diet of antirachitic potency practically equal to that of the acid-treated oatmeal diet with the same phosphorus content.

Confirmation of this view is afforded by comparison of the oatmeal diet with two others which may be regarded as "artificial oatmeal" diets. In the first of these the cereal was maize, with nearly the same proportion of inositolhexaphosphate but with less total phosphorus, and this was brought up to the same level as in oatmeal by the addition of disodium hydrogen phosphate. In the second diet the low phosphorus content of the maize+white flour mixture (estimated at total P, 0·17 %; phytin-P, 0·11 %; other P, 0·06 %) was raised by the addition of sufficient sodium inositolhexaphosphate (containing a little phosphate) to give a mixture (total P, 0·41 %; phytin-P, 0·32 %; other P, 0·09 %) comparable with the oatmeal (total P, 0·41 %; phytin-P, 0·32 %; other P, 0·09 %). The values of ϕ for these two diets, relative to the oatmeal diet, are 1·1 and 0·84 respectively. They are in fact equal to that of the oatmeal diet within the limits of experimental error. It is therefore concluded that, under these conditions, the differences in antirachitic potency between oatmeal and either maize or a maize+white flour mixture (20 : 48) can be practically entirely accounted for by the differences in content of phosphorus in available forms and in the non-available form of inositolhexaphosphate. E. Mellanby [1930], referring to the cereal effect in rickets, said "a greater deficiency of calcium and phosphorus in the body can be brought about by diets which actually contain more of these substances, so that an analysis of mineral intake may be quite misleading as to the efficacy of a diet in adequately supplying these substances to the tissues." The work now described in this paper is in full accordance with this view, in that it has shown that in high calcium diets cereals are apparently more rachitogenic than other material of the same mineral composition, just because their phosphorus content is illusory—the diet is deficient in available phosphorus in spite of being rich in total phosphorus.

Results with low calcium diets.

It is to be regretted that the above experiments are necessarily confined to rats on a high calcium-low phosphorus type of diet, the only conditions under which methods of quantitative comparison of calcifying or anticalcifying action have been worked out. However, the results of the preliminary experiments with rats on low calcium-high phosphorus diets described in this paper, although by no means definitive, give an indication that inositolphosphoric acid has, under such conditions, an anticalcifying action comparable with that of phosphoric acid for the same amount of phosphorus. It is possible to assume that this action is the result of lowering the availability of the calcium by binding it as a calcium salt which is at least as insoluble as calcium phosphate.

Inositolhexaphosphoric acid forms a calcium salt which is sparingly soluble in water, but exact data of its solubility relations are lacking. Under physiological conditions, the experiments of Starkenstein [1914] indicate that the toxicities of orthophosphates and inositolphosphates, which are attributed to their powers of precipitating calcium, are equal in solutions of equal normality ($M/3$ and $M/12$, respectively), when they are administered by mouth or subcutaneously, but that inositolhexaphosphate is three times as toxic as orthophosphate when injected intravenously. Starkenstein suggests that conversion of calcium into a non-ionised form is a factor in this toxic action. In low calcium diets there is thus, in addition to the factor of the low availability of the phosphorus, this fact that inositolhexaphosphoric acid is a precipitant of calcium and tends to reduce the latter to a deficiency level. The exact extent of this tendency is not susceptible of measurement by the methods we have been able to employ, but evidence from other sources shows conclusively that an effect of this kind exists.

The cereal factor in calcification.

It is of particular interest to discuss further the experimental results of M. Mellanby [1929]. The evidence presented with respect to the modifying influence of certain forms of treatment on cereals shows a remarkable parallelism with the known effect of similar treatment on inositolphosphates. The destruction of the anticalcifying factor by boiling oatmeal with 1 % hydrochloric acid, first demonstrated by Green and Mellanby [1928], and its relative stability to sodium hydroxide are comparable with the relative rates of hydrolysis of inositolphosphate by acid and alkali [Plimmer, 1913, 2]. The conclusion that the destruction of the anticalcifying factor during germination and malting may be enzymic is in accordance with the known occurrence of phytase in malt [Adler, 1916]. Further, the possibility of association of the anticalcifying factor with cereal proteins, indicated by digestion experiments on the proteins of oatmeal, recalls the combination of inositolphosphate with protein substances in oatmeal demonstrated by Lindenbaum [1926; *cf.* Koehler, 1926; Mnich, 1931]. It would be of interest to extend experimentally the correlation of anticalcifying properties with the relative proportions of inositolhexaphosphate and phosphate, already demonstrated in this paper in the case of acid treatment of oatmeal.

Vitamin D testing.

The distinction between phytin-phosphorus and other phosphorus compounds in cereals with respect to availability in rachitogenic diets is of significance in connection with certain aspects of vitamin D testing. It has been a common experience in this and other laboratories that unexplained variations in the severity of rickets produced by a rachitogenic diet occur from time to time in spite of a reasonably careful control of the diet [*cf.* Bourdillon *et al.* 1931]. One possible factor in this variation may be variations in the proportions of phytin and other phosphorus in samples of maize of similar total phosphorus content. Moreover, since phytase is present in the grain, the proportion of inorganic phosphate may increase on prolonged storage [*cf.* Minkovska, 1926].

Nervous degeneration.

The word "toxamin" was applied by E. Mellanby to "substances [in cereals] of unknown composition which have harmful effects in the body and can be antagonised by specific vitamins" [E. Mellanby, 1930]. As well as an anticalcifying toxamin, toxamins producing nervous symptoms are believed to be present

in cereals. It seemed worth while investigating whether inositolhexaphosphoric acid, which in these experiments accounted for the anticalcifying toxamin, also played some part in the production of nervous degeneration. This idea was supported, during the course of this work, by the appearance of a paper by Stockman and Johnston [1933], in which they described the extraction from cereals of an acid material which produced symptoms of nervous degeneration in monkeys. It seemed likely, considering their methods, that the material contained inositolhexaphosphoric acid, and this led us to try the effect of sodium inositolhexaphosphate on monkeys. The results obtained have so far been negative and are given in summary form only. Three female *Rhesus* monkeys were used, weighing 2·7–3·1 kg. They were kept under observation for a preliminary period of 3 months and were in good condition when the experiment was started. The monkeys were then dosed by stomach tube. One monkey, which served as control, was given a daily dose of 1 g. of sodium chloride in 100 ml. of water. Doses of 5–10 g. of sodium inositolhexaphosphate in 100 ml. of water daily given to the other two produced only severe diarrhoea and sometimes vomiting. After 2 weeks, the dose was reduced to 1·25 g. and 1·5 g. in 100 ml. daily. At this level no toxic symptoms were observed even after 3 months. For the first 2 weeks of this period, normal diet was continued, after this the intake of calcium and of vitamin A was restricted, and the proportion of cereal (wheat, white maize and rice) was greatly increased.

At no stage in the experiment was there any significant difference in behaviour or growth between the control monkey and the experimental animals. It seems evident that sodium inositolhexaphosphate is not the primary cause of the nervous degeneration and other toxic effects observed by Mellanby and by Stockman and Johnston.

Summary and conclusions.

A method of quantitative comparison of the influence of different cereal diets of the high calcium-low phosphorus type has been applied to the comparison of oatmeal, a maize + white flour mixture and maize.

In the type of diet used, containing a great excess of calcium, the replacement of maize + white flour by oatmeal causes a slight healing of rickets. The concomitant increase in phosphorus content might be assumed to account for this, but when the total phosphorus contents of the two diets are equalised by the addition of disodium hydrogen phosphate to the maize + white flour diet, the healing effect of the latter is considerably greater than that of the oatmeal diet.

This can be accounted for by the fact that a large proportion of the phosphorus present in oatmeal is actually in the form of inositolhexaphosphoric acid, which has been shown to be a poorly available form.

After treatment with 1 % hydrochloric acid the oatmeal diet has an antirachitic potency equal to that of the maize + white flour + phosphate diet, and the hydrolysis of inositolhexaphosphoric acid which takes place in this process affords sufficient explanation of the increased potency.

A series of quantitative comparisons has shown that the differences between oatmeal, maize + white flour, and maize can be completely accounted for by differences in the total phosphorus content and in the proportion of inositolhexaphosphoric acid.

In diets of high calcium content the apparent rachitogenic effect of cereals when compared with other material of the same phosphorus content is due to the fact that the cereal-phosphorus is not in an available form.

These experiments are, it should be emphasised, concerned only with high calcium-low phosphorus diets. Where defect in calcium is the factor limiting calcification, there are grounds for believing that excess of inositolhexaphosphoric acid will interfere with the absorption of calcium in the same way as excess of phosphoric acid. The same quantitative methods used with the high calcium diets cannot be applied here, but preliminary experiments have shown that diets containing excess of phosphate or of phytate are about equal in anti-calcifying power.

The relative proportions of phytin-phosphorus and other phosphorus in cereals is of significance in diets used for vitamin D testing.

Administration of sodium inositolhexaphosphate by mouth to monkeys produced no specific nervous symptoms. Thus it appears that this substance is not related to the toxamins of cereals which produce nervous degeneration.

A preliminary note on the subject of these investigations has been published previously [Bruce and Callow, 1932].

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LXXVI. THE PHOSPHATASE ACTIVITY OF SPLEEN EXTRACTS.

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THERE have been many references in the literature to the presence of enzymes, capable of synthesising and hydrolysing phosphoric esters, in mammalian tissues. Grosser and Husler [1912] and Forrai [1923] described such an enzyme in the intestine. Robison [1923] described similar enzymes in the bone, kidney, liver, spleen and pancreas and in this and a subsequent communication [Robison and Soames, 1924] suggested a possible function for the enzyme occurring in the bone in relation to bone formation. This suggestion has received support, and much evidence, which has been clearly summarised by Kay [1931], has accumulated in its favour.

Kay [1928, 1; 1931] has concluded that the phosphatases of the bone, kidney, intestinal mucosa and blood-plasma are identical, basing his claims upon the following evidence.

1. The optimum hydrogen ion concentration for the phosphatases of the bone, kidney, intestinal mucosa and the blood-plasma is the same. Thus for each of these tissues, for primary esters of phosphoric acid it is 8·9 [Kay, 1926; 1928, 1], for secondary esters it is 7·2–7·6 [Asakawa, 1929] and for pyrophosphoric acid it is 7·6 [Kay, 1928, 2].

2. All these enzymes hydrolyse β-glycerophosphoric acid more rapidly than the corresponding α-compound [Kay and Lee, 1931].

3. The dissociation constant of the enzyme substrate complex, using sodium β-glycerophosphate as substrate, is the same for all the enzymes under discussion [Michaelis and Menten, 1913, quoted by Kay, 1932].

4. Extracts of these tissues, *i.e.* bone, kidney and intestine, will synthesise phosphoric esters under identical conditions [Kay, 1926; Jenner and Kay, 1931; Roche, 1931].

5. Magnesium ions activate the phosphatases from these tissues under very similar conditions [Jenner and Kay, 1931].

Martland *et al.* [1924] reported the presence of a phosphatase in the red cells. Roche [1931], working in Robison's laboratory, further substantiated this claim by separating the plasma and red cells of whole blood and comparing the phosphatase properties of the two fluids under strictly comparable conditions. He demonstrated differences in the properties. Thus the optimum hydrogen ion concentration for the phosphatase of the red cell was found to be p_H 6·0 as compared with p_H 8·9 for the enzyme of the plasma. Moreover the red cell-enzyme hydrolysed α-glycerophosphoric acid more rapidly than the corresponding β-compound, while the plasma-enzyme hydrolysed the β-compound more rapidly.

Robison and Soames [1924] described experiments upon the p_H-activity of the bone, kidney, spleen, liver and pancreatic extracts. They interpreted their

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results as evidence in favour of the hypothesis that the spleen, liver and pancreas contained a phosphatase different from that of the bone and kidney.

In the present work this suggestion has been more fully investigated, and evidence is adduced to suggest that the liver and spleen contain a phosphatase which possesses properties somewhat different from those of both the bone- and the red cell-enzyme.

Technique.

The tissues used in the following experiments were generally from sheep or bullocks and were obtained from the public slaughterhouse immediately upon the death of the animal and used without delay in the majority of cases. Wherever delay occurred the tissue was stored at 0° until ready for use.

In the case of the dog tissue, I am indebted to Dr A. Hemingway, of the Physiology Department, of the University College, Cardiff, for his generosity in supplying me with tissues from the animals which he was using for other experiments. These animals were killed under anaesthetic, and the tissues were obtained immediately. There was no pre-death treatment which might invalidate the results here reported.

Extracts of tissue were prepared by a method similar to that of Kay [1928, 1]. The tissue was minced and then ground up with sand and chloroform water. In the case of the spleen and liver an active suspension was obtained by grinding up 1 part of tissue with 5 parts of chloroform water. After standing overnight at room temperature, the mixture was filtered through glass-wool and used without further purification.

The general method of the research consisted of buffering the extract with phthalate, borate or glycine buffers to a suitable p_H and then measuring the extent of hydrolysis of α- and β-glycerophosphoric acids at various p_H values and after heating at 37°. In order to control the p_H more efficiently it was found necessary to use three glycerophosphate solutions, the p_H of which had been previously adjusted for use over small acid, neutral or alkaline ranges. Even under these circumstances it was found that the buffer solutions did not accurately maintain solutions containing tissue extract at the nominal hydrogen ion concentration of the buffer used. Consequently it was always found necessary to carry out a p_H determination in conjunction with each phosphorus analysis; p_H was measured by the capillator method.

Inorganic phosphate was estimated by the method of King [1932]. Furthermore in view of the anomalies that glycerophosphate can cause in the determination of inorganic phosphorus [Davies and Cule-Davies, 1932], both the amount of substrate used and the time taken for colour development were carefully controlled as suggested by these authors.

The variations in the phosphatase activity of spleen and liver extracts at various hydrogen ion concentrations.

Table I shows the variation in the extent of hydrolysis of sodium β-glycerophosphate with extracts of spleen and liver at varying hydrogen ion concentrations.

Each tube contained 5·0 ml. of buffer solution, 1·0 ml. of tissue extract and 1·0 ml. of 1·5 % sodium β-glycerophosphate solution. The tubes were heated for 4 hours at 37° and then proteins were removed by the addition of 1·0 ml. of 20 % trichloroacetic acid. Aliquot portions of the protein-free filtrate were taken for analysis.

Table I. *The variation in phosphatase activity of tissue extracts under varying conditions of* p_H.

mg. of P produced

p_H	1·0 ml. of spleen extract	1·0 ml. of liver extract	1·0 ml. of kidney extract	2·0 ml. of plasma	2·0 ml. of lysed red cells
4·2	0·280	0·280	0·115	—	—
4·6	0·346	0·290	0·115	—	0·110
4·8	—	—	0·115	—	—
5·0	0·346	0·260	—	—	0·122
5·2	0·190	0·220	0·110	—	—
5·6	0·126	—	—	—	0·147
6·4	—	0·175	—	0·070	0·206
6·8	—	0·165	0·090	0·130	0·110
7·0	0·046	0·150	—	—	0·125
7·4	—	—	—	—	0·125
7·6	0·046	—	0·258	0·360	—
8·0	—	0·256	—	0·580	—
8·3	0·126	0·320	0·381	0·606	—
8·9	0·250	0·400	0·461	—	—
9·4	0·260	0·500	0·474	—	—
9·6	0·230	0·450	—	—	—

The above figures are corrected for the amount of P initially present in the enzyme preparation. The zero hour controls were spleen, 0·360 mg.; liver, 0·240 mg.; kidney, 0·075 mg.; plasma, 0·070 mg.; red cells, 0·052 mg. P per volume of tissue extract *etc.* indicated in the table.

The plasma and lysed cells were heated at 37° for 20 hours.

From p_H 4·4 to 5·0 extracts of spleen show an increasing activity reaching a maximum about p_H 4·8. At less acid reactions, the activity decreases fairly rapidly reaching an almost negligible value at p_H values near neutrality. From p_H 7·6 an increase of activity occurs and the rate of increase is fairly rapid. A second maximum is reached at or near p_H 9·0.

Liver extracts exhibit the same phenomenon. The activity increases to a maximum about p_H 4·7 and then decreases to a minimum at p_H 7·0, finally reaching a maximum again at p_H 9·0.

For purposes of comparison and control, similar series of observations were carried out with kidney extracts and red cells. These results are incorporated in Table I.

Kidney extract was prepared by the method of Kay [1926] in 5 % suspension. Plasma and cells were separated by centrifuging. The plasma was centrifuged a second time in order to ensure the removal of all traces of blood cells. The cells were washed three or four times with isotonic saline and then lysed with an equal quantity of distilled water. They were allowed to stand at room temperature for 12 hours in order to facilitate hydrolysis of naturally occurring esters of phosphorus.

Kidney extracts showed only one optimum of activity and that at p_H 9·0. Plasma exhibited phosphatase activity only on the alkaline side of neutrality. The red cell-enzyme, however, exhibited its maximum effect on the acid side of neutrality. These results confirm the findings of other workers [Kay, 1926; 1931; Asakawa, 1929; Roche, 1931].

These experiments demonstrate the fact that liver and spleen extracts exhibit two optima of hydrogen ion concentration for phosphatase activity. One occurring at p_H 9·0 and the other at p_H 4·5 to 5·0. This property is characteristic of liver and spleen but not of kidney, plasma or red cells.

The relative activities of spleen extracts at the two optima of hydrogen ion concentration.

The relative activities of spleen extracts at p_H 5·0 and 8·8 are not constant, either from animal to animal in the same species or from species to species (Table II).

Table II. *The relative activities of extracts of the spleen of various animals at* p_H *5·0 and* p_H *8·8.*

$$\text{Ratio} = \frac{\text{Activity at } p_H\ 5{\cdot}0}{\text{Activity at } p_H\ 8{\cdot}8}.$$

Animal				
Bullock	Calf	Pig	Dog	Lamb
2·30	1·00	9·00	2·00	2·32
2·80	2·70	4·00	2·90	2·09
2·70	4·50	6·00	—	1·48
0·80	4·30	3·20	—	2·26
2·20	1·40	5·50	—	—
1·50	—	1·40	—	—
—	—	5·50	—	—
—	—	3·80	—	—

The most consistent results were found with bullock, lamb and dog spleens. The average value found for 6 bullocks' spleens was 2·1, with maximum and minimum values of 2·8 and 0·8 respectively. The values found for 2 dogs' spleens were 2·0 and 2·9. The average value for the spleens of four lambs was 2·0, with the maximum and minimum values of 2·3 and 1·5.

The variation of this ratio was however most marked in calf and pig spleens. Thus the maximum and minimum values in the spleens of 5 calves were 4·5 and 1·0 and the average was 2·8. Its deviation was even greater with pigs' spleens. In this species the maximum and minimum values were 9·0 and 1·4 respectively, with an average of 6·0.

The effect of magnesium ions upon spleen extracts and red blood cells under varying conditions of hydrogen ion concentration.

Erdtmann [1927; 1928] and Jenner and Kay [1931] claim that magnesium ions exert an activating effect upon kidney- and bone-phosphatase and also upon the red cell-enzyme at p_H 7·6. The effect of magnesium ions upon spleen extracts and also upon the red cell-enzyme has been observed under different conditions of hydrogen ion concentration and compared with the corresponding effect on the bone-enzyme. The results are incorporated in Fig. 1 and demonstrate the effect of 0·006 *M* magnesium chloride upon spleen- and red cell-phosphatase. The broken line curves show the variation in activity of spleen extracts and also lysed red cells in the presence of added magnesium; the continuous line curves the behaviour in the absence of the added substance.

The observations with spleen-phosphatase yielded an unexpected result. The maximum relative effect occurred at p_H values near the neutral point. From p_H 4·5 to 5·5 a slight inhibition occurred and from p_H 6·0 to 9·5 a definite stimulation, although the maximum relative effect occurred between p_H 6·0 and 8·0. Table III illustrates this point.

Similar results were observed with red blood cells. The maximum effect was observed at p_H values near neutrality (see Fig. 1). Plasma, kidney and bone

Table III.

p_H	mg. P produced in the presence of added Mg	mg. P produced in the absence of added Mg
5·0	0·225	0·264
7·4	0·100	0·021
8·9	0·200	0·084

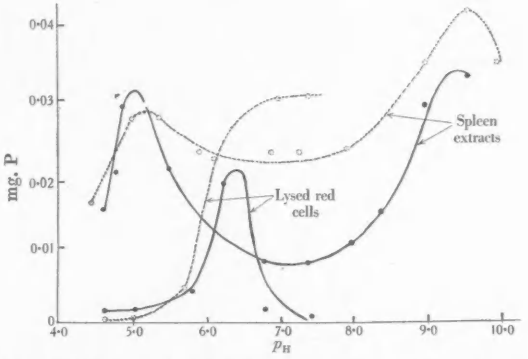

Fig. 1. The hydrolysis of β-glycerophosphate in the presence and absence of added magnesium, by spleen extracts and lysed red cells under varying conditions of hydrogen ion concentration.

•——• Hydrolysis in absence of added Mg; o - - - - o Hydrolysis in presence of added Mg.

extracts all show marked stimulation by magnesium, particularly with dialysed preparations. The increase in activity varies with the p_H, exhibiting a maximum at the optimum p_H of the enzyme.

Both with blood and spleen, the effect of magnesium seems to be to move the optimum p_H of the enzyme to more physiological values and to facilitate the breakdown of organic phosphates at conditions near neutrality. Inasmuch as this is true, we have a fundamental difference between the enzyme of the blood and spleen on the one hand and that of the bone, kidney *etc.* on the other. The combined enzyme-magnesium complex of the bone-enzyme functions optimally at p_H 8·9, whilst that of the spleen and blood exhibits its maximum effect at or near neutrality.

The hydrolysis of α- and β-glycerophosphates by spleen extracts.

The rates of hydrolysis of α- and β-glycerophosphoric acids in the presence of the bone- and red cell-enzymes are different. The bone-enzyme hydrolyses β-glycerophosphoric acid more rapidly than the α-isomeride [Kay, 1926; Kay and Lee, 1931]. The red cell-enzyme however hydrolyses the α-compound the more rapidly [Roche, 1931].

Fig. 2 shows the variation in the rates of hydrolysis of α- and β-glycerophosphoric acids under various conditions of hydrogen ion concentration, using spleen extracts as the source of enzyme. The β-compound was used as the sodium salt: the α-isomeride was used as the ammonium salt. Solutions were prepared to contain the same concentration of phosphorus.

The α-isomeride was hydrolysed in a similar manner to the β-compound, in that the p_H-activity curve exhibited two optima at the same p_H values as with the β-compound as substrate. From Fig. 2 it will be seen that the ratio of

substrate hydrolysed at p_H 5·0 to that at p_H 9·0 varies with the substrate. Thus with the β-compound the ratio was 0·8 and with the α-compound, using the same extract and at the same time, the ratio was 1·7.

The relative rates of hydrolysis of the two compounds were however quite different. The β-compound was hydrolysed rather faster than the α-compound at alkaline p_H values. Near neutrality, the two substances were hydrolysed at

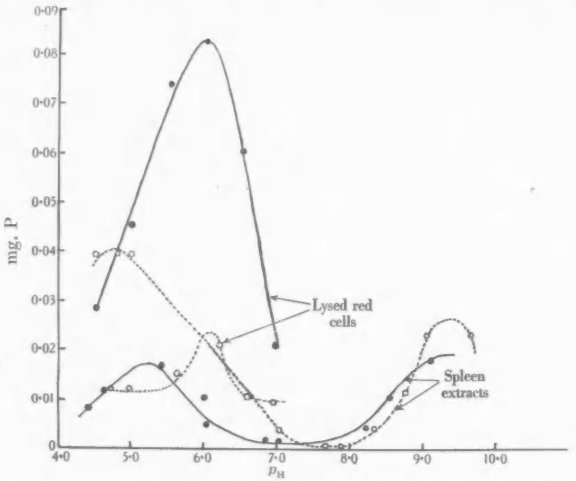

Fig. 2. The hydrolysis of α- and β-glycerophosphates at varying hydrogen ion concentrations, by spleen extracts and lysed red cells.

●——● α-Glycerophosphate; ○- - - -○ β-Glycerophosphate.

nearly the same rate. On the acid side of neutrality however, the β-compound was hydrolysed about three times as fast as the α-compound. In order to control this result the experiment was repeated with red cells. Roche's results were substantiated and a marked difference in the properties of the two enzymes was emphasised (Fig. 2).

The effect of magnesium upon the rate of hydrolysis of α-glycerophosphoric acid was the same as upon that of the β-acid. The relative difference in the rates of hydrolysis was unaltered.

Discussion.

The identity of enzymes cannot be determined exactly as long as one enzyme is mixed with others of the same group. If it is possible to separate the group into its components, then the problem becomes much simpler and the results more definite. The identity of the phosphatases occurring in mammalian tissues cannot be accurately defined, because although results have been quoted in favour of the identity or otherwise of the various phosphatases from animal sources, such results have been based upon the evidence of experiments in which either crude tissue extracts or mixtures of enzymes have been used.

Up to the present, all the reliable evidence suggests the existence of but two distinct and separate phosphatases. (i) The bone-enzyme and (ii) the red blood cell-phosphatase. Roche's claim that the red cell-enzyme is a different mechanism

from that of the bone-phosphatase is a strong one inasmuch as he was able to effect a separation of the enzymes of bone and the red cell and to demonstrate a difference between their properties.

In the present communication experiments have been described which demonstrate the fact that spleen extracts are capable of exhibiting phosphatase activity upon both the acid and alkaline sides of neutrality. The interpretation of such results is difficult because it has not yet been possible to purify the extracts to any great degree, nor has it been possible to separate the extracts into fractions which contain two distinct enzymes. Even so the assumption that spleen extracts contain two distinct and separable enzymes, each capable of hydrolysing phosphoric esters, may be made, in so far as it explains all the facts at our disposal.

Thus such a hypothesis explains the existence of two optima of p_H, the fact that the ratio of the activities of the extracts at the two optima is such a variable quantity (if we are dealing with the same mechanism over the whole range of hydrogen ion concentration studied, then it is reasonable to believe that the ratio should be a constant one); and lastly it explains the effect of magnesium.

The two enzymes may be described for the sake of clarity as the "acid" and "alkaline" phosphatases of the spleen. A comparison of the properties of these two enzymes with those of the bone and blood cells shows some interesting similarities and differences (Table IV).

Table IV. *Comparison of properties of the phosphatases of spleen, bone and red cells.*

	Source of enzyme			
Property	Bone	Red cell	Spleen, acid	Spleen, alkaline
Optimum p_H	8·9	6·0	4·5–5·0	9·0
Effect of magnesium at optimum p_H	Stimulation	Stimulation	Slight inhibition	Stimulation
Effect of magnesium at p_H 7·6	Slight stimulation	Maximum stimulation	Maximum stimulation	Slight stimulation
Relative rates of hydrolysis of α- and β-glycerophosphates	Hydrolyses β-compound 30 % faster than α	Hydrolyses α-compound 300 % faster than β	Hydrolyses β-compound 300 % faster than α	Hydrolyses β-compound about 30 % faster than α

As a result it is possible to identify the alkaline enzyme of the spleen with the bone-enzyme. Thus they both possess the same optimum p_H. They also hydrolyse α- and β-glycerophosphates at the same relative rates, and the effect of magnesium is the same upon both, at both the optimum p_H and at p_H 7·6. The acid enzyme of the spleen however, exhibits markedly different properties from either the alkaline enzyme or the bone-enzyme (Table IV). It resembles the red cell-phosphatase but also differs from this chiefly in two respects; it possesses a different optimum p_H, and it hydrolyses α- and β-glycerophosphates at different rates from the red cell-phosphatase.

The fact that the acid enzyme of the spleen differs from the red cell-phosphatase in just those properties which Kay used for grouping the phosphatases of the bone, kidney *etc.*, suggests the possibility that the two mechanisms may be different. Since, however, little is known of the effect of naturally occurring stimulants and retardants to phosphatase activity it must be remembered that possibly the differences here described may be due to the effect of such substances.

SUMMARY.

1. Extracts of spleen and liver show two optima of hydrogen ion concentration for phosphatase activity. Extracts of bone and intestine and blood-plasma and red cells show only one optimum.

2. The two optima for spleen and liver extracts occur at p_H 4·5–5·0 and at p_H 8·9.

3. There is no relationship between the phosphatase activity at the two optima either from animal to animal or from species to species.

4. Magnesium ions stimulate spleen-phosphatases from p_H 6·0 to 9·5. Below p_H 6·0 a slight inhibition is produced.

5. The maximum relative effect of magnesium ions is found at p_H values near neutrality.

6. Spleen extracts hydrolyse β-glycerophosphoric acid at p_H 5·0 about three times as rapidly as the corresponding α-compound.

7. It is suggested that the spleen contains two separate and distinct phosphatases, one which is identical with the bone-enzyme and the other which possesses properties different from those of the enzyme of either bone or the red cell.

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LXXVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XII. THE ACTION OF PHENYLHYDRAZINE ON THE PASTEUR REACTION AND ON TISSUE RESPIRATION.

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The most pronounced feature of the metabolism of the cancer cell is its property of aerobic glycolysis. With the possible exception of a few spontaneous mouse tumours, where the metabolism of the cancer cells may be masked by that of the connective tissues, all tumours yet examined continued to form lactic acid under aerobic conditions [Warburg, 1930]. The aerobic glycolysis is an expression of a deficiency in the tumour respiration, characterised by a lowering of the R.Q. of the tumour tissue to a level below the carbohydrate value of R.Q. found in normal tissues with similar powers of glycolysis [Dickens and Šimer, 1930, 2; 1931, 2] whether or not the total respiration is diminished in extent. The appearance of aerobic glycolysis shows that in such tissues the mechanism, called by Warburg the Pasteur reaction, which normally correlates respiration and glycolysis, is defective; for normally in a wide variety of aerobic animal and vegetable cells there is a virtually complete suppression in oxygen of the formation of products of the cleavage type of metabolism.

There are two methods by which this problem can be more closely investigated; namely either by the study of methods of artificial production of the property of aerobic glycolysis or by attempts to restore the damaged metabolism to normal. The second of these possibilities is the more difficult to achieve and experiments on this point are in progress.

The most comprehensive study of the conditions under which aerobic glycolysis may be artificially produced is due to Warburg, whose summarised results are as follows [see Warburg, 1929]. I. Inhibition of the respiration; reversibly by means of HCN, irreversibly by temporary asphyxia by keeping the tissue in nitrogen. Spontaneous loss of respiration associated with loss of nuclear material (red blood cell) and accompanying a change of cellular type (embryonic lens). II. Inhibition of the Pasteur reaction, reversibly by ethyl *iso*cyanide, irreversibly by heat and, partially, by exposure of certain embryonic and germinal tissues to salt solutions. To these we may now add certain agents which, like ethyl *iso*cyanide, may be classed under the rather comprehensive title of "heavy metal reagents": 1-amino-2-naphthol-6-sulphonic acid [Krah, 1930], and also reduced glutathione which, according to Bumm and Appel [1932] specifically and reversibly inhibits the Pasteur reaction.

Warburg [1926] pointed out that the poisoning action of ethyl *iso*cyanide on the Pasteur reaction favours the theory of a heavy metal catalyst; the metals

in consideration being iron or, less probably, manganese. Since, on this theory ethyl *iso*cyanide reacts with the catalyst of the Pasteur reaction but not with the respiratory catalyst, whilst the same compound is without action on methaemoglobin but reacts with haemoglobin, the further view that the catalyst of the Pasteur reaction may contain iron in the ferrous state was tentatively suggested. Krah concludes from the action of the reagent mentioned that he has demonstrated the ferrous nature of the catalyst.

In the present paper the action of phenylhydrazine on tissue metabolism is investigated. Phenylhydrazine belongs, according to Lipschitz [1924] to the class of "indirect blood poisons" which are able to oxidise blood pigments only in the presence of oxygen, becoming themselves oxidised in the process. Warburg *et al.* [1931] showed clearly that in oxygen, but not in inert gas (CO), erythrocytes treated with phenylhydrazine become brown in colour, without spectroscopically detectable methaemoglobin formation, whilst on haemolysis there is a heavy deposition of denatured globin, whose production is presumably accompanied by liberation of haematin; for on reduction in an atmosphere of CO the amount of gas taken up by the phenylhydrazine-treated cells was equal to their loss of oxygen capacity. On shaking these cells with air the spectrum of methaemoglobin slowly appears owing to the catalytic action of the haematin on part of the free haemoglobin, and by the reduction of this methaemoglobin sugar can be oxidised in the cells. The treated cells thus show an ability to oxidise glucose which is absent from untreated ones, this property being dependent on the high content of haemoglobin in the starting material. Such an increased sugar oxidation could not therefore be expected to occur in other cells. On the other hand it was of importance to test the reaction upon ordinary body cells of this substance which reacts in the presence of oxygen with compounds such as haemoglobin containing ferrous iron, destroying the complex and causing denaturation of the associated protein. A substance possessing these properties might be expected to influence the Pasteur reaction, since reagents which react within the cell with ferrous iron (ethyl *iso*cyanide, heavy metal reagents of Krah) or denaturation of protein (heat, salt solutions) are known to interrupt the connection between respiration and glycolysis.

In the present paper it is shown that phenylhydrazine, in low concentration, acts as what may be termed an "indirect cell poison," in that in the presence of oxygen, but not in its absence, it is capable of bringing about an aerobic glycolysis which can rise to the anaerobic level. Its action is irreversible, and the substance is without appreciable effect on the anaerobic glycolysis. Whilst with some tissues the respiration is little affected, or even slightly increased, with others it is inhibited to a greater or less extent. The significance of this will be discussed after the description of the experiments.

Experimental.

Methods. The tissue, usually of the rat and in thin razor-slices where necessary, was well rinsed with three changes of Ringer solution, to remove as much of the loosely adhering blood as possible, and was suspended in glucose-bicarbonate-Ringer solution at room temperature and containing sufficient of the $M/20$ solution of phenylhydrazine described below to give the final concentration stated (usually $2{\cdot}5 \times 10^{-3} M$). A control portion of the same tissue was suspended in the untreated Ringer solution. Oxygen containing 5 % CO_2 was allowed to bubble briskly through the suspensions; this pre-treatment usually lasted for 15 minutes. The tissue was then removed, well rinsed first in salt solution,

afterwards for a few minutes in bicarbonate—or phosphate—Ringer while passing the appropriate gas mixture (O_2, or 5 % CO_2 in O_2). It was then transferred to the manometer-vessels containing bicarbonate—or phosphate—Ringer solution with 0·2 % glucose.

Phenylhydrazine. The colourless, recrystallised hydrochloride (B.D.H. "A.R." 73 mg.) was dissolved in 5 ml. water, and made up to 10 ml. with salt-solution and the addition of 0·5 ml. *N* NaOH. This solution (*M*/20) was prepared freshly for each experiment.

Measurement of metabolism. For short experiments, and where the course of the metabolism with time was to be followed, Warburg's [1924] two vessel method was used. Unfortunately with this method not only must a value of R.Q. be assumed in the calculation of aerobic glycolysis, but with increasing value of aerobic glycolysis relative to respiration, the values of the observed pressure changes in the two vessels become more nearly equal, and it follows that the difficulty of obtaining accurate values of respiration with such tissue becomes increasingly great. The technical difficulty of obtaining an accurate measure of respiration in bicarbonate media presents a serious problem with such tissues. To supplement the respiration figures, where the material available was sufficient, the methods of Dickens and Šimer [1931, 1] and Dickens and Greville [1933, 3] have been used. Both need a fairly prolonged experimental time, but the values of respiration, and with the second method of glycolysis, obtained are free from the objection inherent in the use of two different tissue-slices for measurement. The value of aerobic glycolysis in some extreme examples of phenylhydrazine-treated tissue is so great compared with the respiration that no existing method is adequate for really accurate measure of their R.Q. in bicarbonate, the whole extent of the manometer scale being used up by the glycolysis before a sufficiently great respiration had occurred. Finally, measurements of respiration in phosphate-Ringer were made by the method of Dickens and Šimer [1930, 1], but these, while free from such disadvantages, are in a less physiological medium, in which the respiration may not always be the same as in the bicarbonate medium used for the measurement of glycolysis. In the experiments with yeast, where the cells were suspended in acid phosphate, with or without potash as CO_2 absorbent in the vessels, these difficulties did not of course arise. In spite of the technical difficulties mentioned, it is believed that the precautions taken were adequate to ensure reliable measurement in all cases, though in the experiments made by Warburg's method with these highly glycolysing tissues importance should not be attached to minor variations in respiration.

The measurements are collected in Tables I, II and III, and will be briefly surveyed.

Table I. *Embryonic tissues.*

		Control			Treated phenylhydrazine: Pre-treatment						
Exp.	Time (mins.)	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Conc. *M*/lit.	Time mins.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Medium
Mouse outer membrane	—	12·9	5·9	—	3 $\times 10^{-3}$	13	—	14·3	20·6	26·0	B
Mouse placenta	—	—	—	—	3 $\times 10^{-3}$	13	—	8·2	5·2	6·5	B
Mouse embryo	—	20·0	14·5	31·0	3 $\times 10^{-3}$	12	30	16·0	22·5	25·0	B
Guinea-pig outer membrane	—	—	—	—	2·5 $\times 10^{-3}$	15	—	12·3	16·0	20·6	B
Rat outer membrane	90	14·1	—	—	3 $\times 10^{-3}$	15	90	9·3	—	—	Phosphate
	60	15·5	6·2	26·0			60	10·2	31·8	28·5	B
Rat outer membrane	40	9·8	3·2	18·7	3 $\times 10^{-3}$	15	40	7·3	17·3	20·0	B
							120	5·5	—	—	Phosphate
Rat embryo (3 mg. dry wt.)	40	11·3	8·4	14·2	3 $\times 10^{-3}$	10	40	14·1	16·2	16·1	B

Table II.

		Control			Treated phenylhydrazine						
					Pre-treatment						
Exp. no.	Time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	conc. *M*/lit.	Time mins.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Medium
					Rat testis						
1	60	10·8	2·5	7·3	$2·5 \times 10^{-3}$ (no glucose)	8	60	9·4	6·3	8·3	B
	60	9·6	3·5	11·0	$2·5 \times 10^{-3}$	5	60	14·1	6·9	—	B
						15	60	10·6	8·4	9·2	B
3	30	12·0	4·3	12·0	$2·5 \times 10^{-3}$ (tissue not washed)	15	30	9·4	2·9	11·6	B
		10·4	3·9	—	$2·5 \times 10^{-3}$ (tissue slightly rinsed)	15	30	10·3	6·3	—	B
4	30	12·8	4·8	11·0	$2·5 \times 10^{-3}$ (tissue in two changes Ph.NH.NH_2)	15	30	9·9	11·0	10·1	B
	100	9·9	3·5	—	$2·5 \times 10^{-3}$		100	6·0	7·0	—	B
5	1st 30	13·3	5·0	10·6	,,	15	1st 30	15·5	8·9	8·6	B
	next 60	10·3	3·3	10·6			next 60	12·3	6·2	8·6	
6	—	—	—	—	,,	15	120	6·5	4·7	6·6	B†, R.Q. 0·80
7	30	10·3	5·5	9·0	,,	15	30	15·1	9·0	9·0	B
8	120	10·6	2·3	—	R.Q. 0·98 ,,	15	80	8·2	6·4	—	B
								8·6	7·7	—	B
								9·6	7·1	—	B
							120	8·8	6·2	8·1	B†, R.Q. 0·90
					Rat liver						
	120	11·8	0·2	3·0	R.Q. 0·83 ,,	15	120	8·2	0·5	2·9	B†, R.Q. 0·88

Abbreviations: B = method of Warburg [1924]; B* = method of Dickens and Šimer [1931, 1]; B† = method of Dickens and Greville [1933, 3]; Phosphate = method of Dickens and Šimer [1930, 1].

Table III. *Jensen rat sarcoma.*

	Control				Treated phenylhydrazine					
Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.		Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Method
30	11·2	24·9	37·5	—	Pre-treatment	30	13·3	40·5	36·5	—	B
					$2·5 \times 10^{-3}$ *M*		12·9	36·2	36·5	—	
90	6·8	17·4	30·2	—	Ph.NH.NH_2	90	5·9	28·6	31·8	—	B†
90	—	—	32·0	—	for 15 mins.	90	5·6	28·0	34·0	0·7	B†
90	8·4	16·7	27·6	—	in all exps.	90	13·0	27·3	—	—	B
							9·7	24·2	27·6	—	B
30	13·3	20·6	35·7	—		30	15·8	32·5	34·8	—	B
150	13·2	18·0	—	0·73		150	9·0	—	—	0·75	B*
							10·3	(25)	—	0·69	B*
150	9·3	—	—	0·70		150	7·6	—	—	0·77	Phosphate

Abbreviations: B = method of Warburg [1924]; B* = method of Dickens and Šimer [1931, 1]; B† = method of Dickens and Greville [1933, 3]; Phosphate = method of Dickens and Šimer [1930, 1].

Effect of phenylhydrazine on the anaerobic glycolysis. The figures given for $Q_M^{N_2}$ in the tables show the effect of phenylhydrazine on the anaerobic glycolysis of a number of tissues. In some cases a slight fall of glycolysis is observed, but the variation is not as a rule much greater than may be found with duplicate pieces of control tissue after similar pre-treatment but without phenylhydrazine. The tables show that on the whole the pre-treatment with phenylhydrazine in the concentration used is without appreciable effect on the glycolysis measured under anaerobic conditions.

Effect of phenylhydrazine on the aerobic glycolysis. In all the tissues used (with the exception of liver, where the glycolysis is small under ordinary conditions) the effect of treatment with phenylhydrazine is to increase the aerobic glycolysis ($Q_M^{O_2}$) very markedly. In most cases $Q_M^{O_2}$ rises to a level very near to the anaerobic value. This is best seen with tissues showing high $Q_M^{N_2}$, such as the embryonic tissues (Table I), and tumour tissue (Table III). As is well known, many of the embryonic tissues show an appreciable glycolysis in Ringer solution which is not observed when the measurements are made in serum or in amniotic fluid: very young embryos may also glycolyse even in these media unless the enveloping membranes are intact [Negelein, 1925]. Even in such cases (Table I) the value of $Q_M^{O_2}$ found in Ringer solution is greatly increased by pre-treatment with phenylhydrazine.

Of tissues with an intermediate degree of glycolysis, testis (Table II) provides a suitable example. Unlike the other tissues studied, for some unknown reason apparently connected with the animals used, testicular tissue of the rat suspended in Ringer solution shows a very variable aerobic glycolysis. Thus Warburg *et al.* [1924] give the following figures (based on R.Q.$=1$) Q_{O_2} $-12{\cdot}3$, $Q_M^{O_2}+7{\cdot}2$, $Q_M^{N_2}+8{\cdot}5$; and a similar though less pronounced aerobic glycolysis was observed by Dickens and Greville [1933, 1]. On the other hand, in earlier experiments Dickens and Šimer [1931, 2] found much lower values of $Q_M^{O_2}$, and these are similar to the figures given in the present paper. At the other extreme, Barron [1930] reports that though manometrically he observed a small acid formation ($Q_M^{O_2}$ $+1{\cdot}2$), chemically (in phosphate) he was unable to detect any aerobic lactic acid formation in salt solutions with the specimens of rat testis used by him. Since in manometric experiments we have invariably observed some aerobic acid production with this tissue, it was considered desirable to compare the manometric readings with chemical estimations of lactic acid formed. As the result (Table IV) of the analysis (Friedemann and Kendall's [1929]

Table IV. *Lactic acid estimations.*

	mg. dry wt. tissue	Time mins.	Manometrically found mg. acid	Chemical estimation mg. lactic acid
	Rat testis			
Control tissue I	13·6	120	0·39	0·45
	14·8		0·42	0·38
Control tissue II	22·9	100	0·69	0·62
	21·1		0·64	0·70
Phenylhydrazine tissue I	13·8	120	0·78	0·70
	15·8		0·68	0·66
Phenylhydrazine tissue II	24·3	100	1·23	1·12
	8·65	180	1·00	1·08
	Jensen sarcoma			
Control tissue	12·17	90	1·22	1·07
	11·43		1·15	0·96
Phenylhydrazine tissue	8·05	90	1·32	1·25
	8·72		1·43	1·14
	8·68	90	1·25	1·32

modification of Clausen's method), some 90 % of the manometrically observed acid appeared as lactic acid. Since this is almost within the limits of error with a tissue of such moderate glycolysis, there does not seem any reason to doubt that this is also a true lactic acid fermentation. Thus there appears to be a real

difference between the behaviour of rat testis as studied by different workers for which no explanation appears sufficient at the present time.

These considerations however do not affect the present experiments, where control and phenylhydrazine portions of the same tissue were always compared side by side. As will be seen from Table II, phenylhydrazine had a similar effect on aerobic glycolysis to that shown by the embryonic tissues; the value of $Q_M^{O_2}$ rises after treatment to a higher level, near or equal to $Q_M^{N_2}$.

A further type was provided by tumour tissue. Well-growing non-necrotic specimens of Jensen rat sarcoma (derived from a stock tumour kindly supplied by Miss Scott, Barnato Joel Research Laboratories, Middlesex Hospital, London) were used in all experiments. All methods of measurement of metabolism showed the same result, the already pronounced aerobic glycolysis shown by the Jensen sarcoma is greatly increased in the phenylhydrazine-treated tissue (Table III) and reaches a value which is, within the limits of error, equal to the glycolysis of the same tissue in absence of oxygen.

Nature of the acid formed. The possibility that the acid formed in the manometric observations might not have been lactic acid has been excluded by analysis. For this purpose rat testis and Jensen sarcoma were used and the manometric readings compared with Clausen estimations of the lactic acid formed in the vessels, the results (Table IV) show that some 90 % of the acid formed is lactic acid.

Conditions affecting the action of phenylhydrazine on the metabolism. (*a*) *Washing.* Since phenylhydrazine is known to react with haemoglobin under similar conditions to those in which it reacts with the cell-ferments, it is not surprising that incomplete washing of the tissue reduces the extent of its action. It has been observed that in several experiments where completely unwashed testis, for example, has been used phenylhydrazine ($2{\cdot}5 \times 10^{-3} M$) had no measurable action on the metabolism. If, however, the same tissue were treated with a second portion of the phenylhydrazine Ringer solution, the usual increase of aerobic glycolysis was produced (Table II). This point has not been further studied, well washed tissues being always used in subsequent experiments.

(*b*) *Time and temperature.* At room temperature the reaction with phenylhydrazine is not instantaneous but is usually complete in 15 minutes. Exp. 2, Table II, performed with rat testis illustrates this point.

The reaction also occurs at 37° and is not dependent on the presence of glucose in the medium as the following experiments show.

Jensen rat sarcoma. Temperature 37°. Control, $Q_M^{O_2} + 23{\cdot}5$; the same tissue with $1{\cdot}7 \times 10^{-3} M$ phenylhydrazine in vessel (result, corrected for autoxidation of phenylhydrazine) $Q_M^{O_2} + 37$.

Rat testis. Pre-treatment without addition of glucose to the Ringer-bicarbonate used: 8 minutes at room temperature:

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Control	−10·8	2·5	7·3
With $2{\cdot}5 \times 10^{-3} M$ phenylhydrazine	− 9·9	6·3	8·3
	− 9·0	6·3	—

(*c*) *Oxidative nature of the reaction.* The following experiments show that the action of phenylhydrazine occurs only in the presence of oxygen.

The pre-treatment was carried out at room temperature in the closed vessels attached to their manometers; these were filled with either nitrogen + 5 % CO_2, or oxygen + 5 % CO_2 and were shaken as usual. The phenylhydrazine solution (0·2 ml. $M/20$) was contained in the side-bulb, while the tissue suspended in

Ringer-bicarbonate-glucose solution (4 ml.) was in the main part. The solutions were first saturated with the appropriate gas mixtures. In the experiments in nitrogen, chromous chloride solution (0·3 ml.) was contained in the central well as oxygen absorbent. The vessels containing solutions and tissue were first shaken for 10 minutes to allow absorption of any residual oxygen in the anaerobic experiments, the phenylhydrazine was tipped in from the bulbs, and the shaking was continued for 15 minutes. The tissue was then quickly removed, rinsed and transferred to the vessels used for measurement of metabolism. It was noted that the colour of small traces of blood present changed to brown in the oxygenated phenylhydrazine portions only.

	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Rat testis			
Control: pre-treatment in $O_2+5\%$ CO_2	−10·3	5·5	9·0
Ph.NH.NH_2: pre-treatment in $O_2+5\%$ CO_2	−15·1	9·0	—
Control: pre-treatment in $N_2+5\%$ CO_2	−12·8	5·5	—
Ph.NH.NH_2: pre-treatment in $N_2+5\%$ CO_2	−12·5	5·8	—
Jensen sarcoma			
Control: pre-treatment in $O_2+5\%$ CO_2	—	—	32·4
Ph.NH.NH_2: pre-treatment in $O_2+5\%$ CO_2	− 9·5	31·0	—
	− 7·7	27·8	—
Ph.NH.NH_2: pre-treatment in $N_2+5\%$ CO_2	− 8·6	19·1	—

Effect of phenylhydrazine on respiration. If the figures for Q_{O_2} shown in Tables I–III are examined, it will be seen that the rise in glycolysis is not accompanied by a corresponding fall in respiration. Occasionally in short experiments the latter appears to be slightly increased; a similar result was observed by Warburg [1926] in ethyl *iso*cyanide poisoning of surviving tissues, but there does not seem to be any explanation at present for this. On the other hand, and particularly in experiments of greater duration, there is usually a small fall of respiration in the phenylhydrazine tissue; the extent of the fall varies, though not entirely regularly, with different tissues and is fairly uniformly found in extended experiments with testis, sometimes with some of the embryonic tissues, and is less pronounced, or absent, with Jensen sarcoma.

In considering these figures there is a suggestive parallelism with the respiration of the same tissues in the absence of glucose from the medium; this effect of glucose-lack has been fully examined in a previous publication [Dickens and Greville, 1933, I]. It was shown that, in experiments extending over some hours, the respiration of testis falls slowly, but that of most of the other tissues used in Tables I–III is less affected by withdrawal of glucose; probably even with different samples of any particular tissue the extent of the fall is to some extent dependent on the state of nutrition of the animal. The respiration of the Jensen sarcoma is practically identical in extent whether glucose is present or not.

This suggested that the action of phenylhydrazine in inhibiting the Pasteur reaction might be connected with a loss of ability of the poisoned tissue to oxidise glucose. This question has been attacked in two ways. Firstly, the value of R.Q. in a few examples of the poisoned tissue was examined. Unfortunately little help could be expected from these measurements, for with the tissues available the fall in R.Q. in absence of glucose is by no means marked and may even be lacking entirely, as was shown by Dickens and Greville [1933, I] in a number of instances. The most favourable example from this point of view was testis, and here the values of R.Q. and respiration both suffered a fall in the longer experiments (Table II) suggesting that carbohydrate oxidation may be reduced.

Similar measurements with Jensen sarcoma showed that neither the R.Q. nor the respiration undergoes any marked change in the poisoned tissue (Table III); here again the result is comparable with the absence of change of these quantities with the untreated tissue in glucose-free Ringer, as has been previously shown.

The most serviceable examples of cells suitable for the demonstration of carbohydrate oxidation are provided by brain grey-matter [Loebel, 1925] and the yeast-cell [Meyerhof, 1925]. In both of these, unlike the tissues already considered, the continuance of the respiration is dependent on the supply of carbohydrate from without.

The following experiments were designed to determine to what extent the oxidation of glucose is influenced in these cells by treatment with phenylhydrazine, since this appeared to be a critical test of how far it is the oxidation of carbohydrate that is specifically interfered with by this reagent.

Table V. *Rat cerebral cortex.*

		Control				Treated phenylhydrazine						
						Pre-treatment						
No.	Exp. time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Conc. M/lit.	Time mins.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Method
1	30	11·1	3·5	—	—	5×10^{-4}	15	12·9	5·6	—	—	B
						10^{-2}	15	3·9	21·4	19·4	—	B
2	40	13·4	2·5	28·0	—	$2·5 \times 10^{-3}$	15	10·3	11·3	24·4	—	—
								8·6	8·5	—	—	B
	90	—	—	—	—	$2·5 \times 10^{-3}$	15	11·6	10·7	24·0	—	B*
	90	—	—	—	—	5×10^{-3}	15	4·4	15·7	—	—	B*
3	60	—	—	—	—	$2·5 \times 10^{-3}$	15	12·2	10·0	27·0	—	—
								9·6	8·8	—	—	B
	120	—	—	—	—	$2·5 \times 10^{-3}$	15	6·4	11·2	—	0·98	B†
	130	—	—	—	—	$2·5 \times 10^{-3}$	15	8·3	—	—	0·96	Phosphate
								5·9	—	—	1·02	Phosphate
4	60	12·3	4·3	15·9	—	$2·5 \times 10^{-3}$	15	9·5	7·0	17·8	—	B
	90	10·7	—	—	—	$2·5 \times 10^{-3}$	15	8·4	—	—	—	Phosphate
	120	12·6	2·3	—	0·98	$2·5 \times 10^{-3}$	15	9·9	7·5	17·0	1·01	B†
5	120	—	—	—	—	$2·5 \times 10^{-3}$	15	5·0	15·2	18·4	—	B†
								5·8	11·1	18·4	—	B†

Abbreviations: B = method of Warburg [1924]; B* = method of Dickens and Šimer [1931, 1]; B† = method of Dickens and Greville [1933, 3]; Phosphate = method of Dickens and Šimer [1930, 1].

Rat cerebral cortex. The experiments on rat brain tissue were carried out just as for the other tissues in Tables I–III. As in the other experiments glucose was present throughout in the media, a necessary precaution here since the respiration of brain tissue quickly suffers in absence of sugar. The results are shown in Table V. As before, it will be seen that the phenylhydrazine pre-treatment is without effect on the anaerobic glycolysis, whilst in some experiments the aerobic glycolysis rises considerably. In all of these, however, the respiration sinks, more or less slowly, and in spite of repeated trials it has not been found possible by action of phenylhydrazine with this tissue to raise the glycolysis to the anaerobic level without the occurrence of a serious fall in respiration. The R.Q. does not appear to be affected by phenylhydrazine pre-treatment of brain, and it is often similarly unaffected by lack of glucose [Dickens and Greville, 1933, 1].

Thus with brain tissue poisoned by phenylhydrazine, as the aerobic glycolysis increases there appears to be a progressive change in the respiratory metabolism towards that shown by the same tissue in absence of glucose. The question therefore arises: is the oxidation of other metabolites similarly affected? Loebel [1925] showed that the respiration of normal brain tissue can be supported

by glucose, lactate or pyruvate. In the following experiment the effect of these metabolites on the respiration of normal and poisoned brain was studied.

Rat cerebral cortex. Control brain. As control, slices prepared from two brains were treated with phenylhydrazine ($2{\cdot}5 \times 10^{-3} M$) for a time insufficient for the metabolism to be affected.

Medium	Substrate	Respiration (Q_{O_2}) 1st hr.	2nd hr.	R.Q.
Phosphate-Ringer	None added	− 8·0	− 2·6	—
	Glucose 0·2%	−11·0	− 9·6	1·03
	dl-Lactate 0·2%	−12·0	−12·0	1·03
	Pyruvate 0·2%	− 9·5	− 9·3	1·30

Bicarbonate-glucose-Ringer: Q_{O_2} −11·2; $Q_M^{O_2}$ +5·3; $Q_M^{N_2}$ +21·5.

Phenylhydrazine brain. Pre-treatment: oxygenation for 15 minutes at room temperature with $2{\cdot}5 \times 10^{-3} M$ phenylhydrazine.

Medium	Substrate	Respiration (Q_{O_2}) 1st hr.	2nd hr.	3rd hr.	R.Q.
Phosphate-Ringer	None added	− 5·5	−2·1	−1·0	—
	Glucose 0·2%	−10·0	−4·0	−0·8	0·99
	dl-Lactate 0·2%	− 8·3	−6·0	−4·0	1·01
	Pyruvate 0·2%	−10·7	−9·6	−8·6	1·32

Bicarbonate-glucose-Ringer (1st 40 mins.): Q_{O_2} −11·8; $Q_M^{O_2}$ +10·3; $Q_M^{N_2}$ +20·0.

These figures show how the respiration changes with time, and from them it is clear that whilst the ability to oxidise glucose and lactate is severely damaged in the partially poisoned brain, the ability to oxidise pyruvate is in no way affected. This result is particularly noteworthy, since it was shown for tumour tissue [Dickens and Šimer, 1930, 2] that whilst glucose and lactate have little effect on the level of R.Q., pyruvate increases this very markedly to above unity (Jensen sarcoma, Rous sarcoma). Except for the fact that the tumour respiration in absence of added substrate does not fall, a property known to be characteristic of brain tissue, the behaviours towards these metabolites of the partially poisoned normal tissue after treatment with phenylhydrazine and of the tumour tissue appear to be very similar or identical. Pyruvate is the only one of these three substances to be readily oxidised by the two types of tissue, in both of which the respiration is damaged in such a way that it is unable to exercise its normal influence on the aerobic glycolysis.

Baker's yeast. The behaviour towards glucose of the respiration of the yeast cells used (Table VI) was found to be essentially similar to that of brain tissue, but the fall of respiration in absence of glucose (about 90 %) in the washed cells showed that the yeast cells were even more dependent than brain on glucose from the medium for their respiration.

Phenylhydrazine pre-treatment of this yeast inhibited its respiration without appreciably affecting its power of anaerobic fermentation. The aerobic fermentation rose, and calculation of the Meyerhof quotient shows that there was only a slight inhibition of the Pasteur reaction, or in other words the poisoning of the carbohydrate respiration ran almost parallel with the appearance of the carbohydrate fermentation. Finally, with the high concentration of phenylhydrazine, poisoning of the respiration was complete, within the limits of error, whilst the fermentation in air became equal to anaerobic (HCN-poisoned) fermentation of the untreated yeast (Table VI).

As with higher cells, this toxic action of phenylhydrazine on the yeast cell is dependent on the presence of oxygen. With yeast the effect is very marked:

Table VI. *Baker's yeast.*

1. Effect of glucose-lack.

Suspension in $M/20$ NaH_2PO_4, oxygenated by bubbling the gas. 0·6 mg. dry wt. of yeast per ml.

Control portion washed three times at the centrifuge with solution of NaH_2PO_4 ($M/20$), glucose, $M/18$.

"Glucose-free" portion washed three times at the centrifuge with solution of NaH_2PO_4 ($M/20$), without glucose.

Both portions made up to suitable volume in the same solutions as used for washing.

	Q_{O_2} (Respiration)	$Q_G^{O_2}$ (Aerobic ferm.)	$Q_G^{N_2}$ (Anaerobic ferm.)
Result: (temp. 25°) Control portion with glucose	54·5	8·5	111
"Glucose-free" portion in glucose-free phosphate	{4 {7·5	{1·5 {7·5	4·5
"Glucose-free" portion on addition of glucose	55·5	10	123

2. Effect of treatment with phenylhydrazine.

Suspension as above of a different batch of yeast from the same commercial source. 0·3 mg. dry wt. of yeast per ml. in glucose-phosphate solution.

Control portion. Washed three times at centrifuge with glucose-phosphate solution. Made up to original volume.

Phenylhydrazine pre-treatment. To the oxygenated yeast suspension at room temperature, sufficient of a neutralised solution of phenylhydrazine hydrochloride to give the concentration stated was added. After 15 mins. centrifuged, washed three times with glucose-phosphate solution, and made up to the original volume.

Result: (temp. 25°):

Concentration of phenylhydrazine M/lit.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
0 (control)	80	23	180
10^{-4}	82	16	—
$2{\cdot}5\times10^{-4}$	68	60	167
10^{-3}	51	91	161
2×10^{-3}	10	145	150
3×10^{-3}	0	163	168
7×10^{-3}	0	175	165

Note. The values of "anaerobic fermentation" were obtained by poisoning with 2×10^{-3} *N* HCN.

mixture of the phenylhydrazine solution with the non-aerated suspension of yeast merely in contact with air but not shaken is not enough to cause the full alteration of metabolism. Saturation with oxygen however quickly leads to the usual result:

Baker's yeast. 1 mg. (dry wt.) per 3 ml. in NaH_2PO_4 $M/20$, glucose $M/18$; 25°. Pre-treatment with concentration of phenylhydrazine stated for 15 minutes.

		Phenylhydrazine pre-treatment						
		Nitrogen (with $CrCl_2$)		Air (not shaken)			Oxygen (shaken)	
Conc. of phenylhydrazine	Control	7×10^{-3}	10^{-3}	7×10^{-3}	3×10^{-3}	10^{-3}	7×10^{-3}	$3\times10^{-3}M.$
Respiration (Q_{O_2})	− 45	−47·5	−46·5	−29·5	−42·5	−47	0	− 6·5
Aerobic fermentation ($Q_G^{O_2}$)	+ 8	+17	+ 6	+39	+24	+ 9	+101	+110
Anaerobic fermentation ($Q_G^{N_2}$)	+108	—	—	—	—	—	+ 93	+109

Other reagents: phenylhydroxylamine and amyl nitrite, both of which lead to methaemoglobin formation in the erythrocyte [Heubner, 1913; Lipschitz, 1924; Warburg *et al.* 1930; 1931] and to increased respiration of the latter in glucose, are without similar effect to that of phenylhydrazine on the Pasteur reaction

Table VII. *Other reagents.*

		Control			Treated tissue				
					Conc. in Ringer				
		Q_{O_2}	$Q_M^{1\cdot2}$	$Q_M^{N_2}$	M/lit.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	Method
1. Rat testis.	Phenylhydroxylamine†	10·0	3·5	8·8	6×10^{-4}	1·3	0·7	9·2, 7·8	B
					$1{\cdot}2 \times 10^{-4}$	7·7	2·6	—	B
	Quinone†	—	—	—	10^{-3}	4·9	1·9	3·1	B
					5×10^{-4}	4·4	1·3	3·3	B
						3·4	—	—	Phosphate
2. Jensen sarcoma.	Phenylhydroxylamine*	16·4	22·8	34·4	10^{-3}	3·9	15·0	30·0	B
	Amyl nitrite†	7·8	19·7	30·4	*ca.* $M/100$	4·2	15·2	24·6	B

Abbreviations: B = method of Warburg [1924]; Phosphate = method of Dickens and Šimer [1930, 1].
* = pre-treatment; † = in vessel with the tissue.

(Table VII), their action being to lessen the aerobic metabolism generally; in depressing Q_{O_2} they are more like the indifferent narcotics. Phenylhydroxlamine was the pure colourless recrystallised substance freshly prepared according to Wohl [1894]. It was dissolved in the Ringer solution in the concentration stated immediately before use; the solution however very quickly undergoes decomposition. Amyl nitrite (B.D.H., A.R.) was also added to the Ringer solution directly and the mixture shaken mechanically for half an hour before use. In both cases corrections for spontaneously occurring pressure changes were applied.

Since these reagents, in their reactions with haemoglobin at any rate, are without effect on the globin, whilst phenylhydrazine brings about its denaturation, it seems likely that it is to this property of protein denaturation that phenylhydrazine owes its action on the Pasteur reaction.

The mechanism of the Pasteur reaction. According to Warburg's conception [see Warburg, 1930], the increase of aerobic glycolysis without a corresponding fall in the respiration is the criterion by which we may judge the specific inhibition of the Pasteur reaction. The extent of the inhibition is to be estimated by the fall in the value of the Meyerhof quotient: (aerobic *minus* aerobic glycolysis)/(respiration). From a normal value of about 2, this quotient will sink to zero when inhibition of the Pasteur reaction is complete.

Judged by this standard, phenylhydrazine is a specific, irreversible inhibitor of the Pasteur reaction only in certain tissues; these are the tissues whose continued respiration is not dependent upon their being provided with carbohydrate from without. On the other hand, in tissues whose respiration is dependent on exogenous carbohydrate, phenylhydrazine acts as a specific poison for the respiratory enzymes, where its effect on the respiration may, as with the yeast cell, resemble that of cyanide in its completeness.

A number of questions are at once suggested by this apparently dual rôle of phenylhydrazine as a poisoner on the one hand of the Pasteur reaction and on the other of the oxidation of carbohydrate. In the first place it does not seem probable that this property possessed by phenylhydrazine of interference with glucose oxidation without interfering with the mechanism of glucose cleavage is limited to tissues whose respiration falls in the absence of glucose. It is therefore a probable assumption, supported by the evidence as far as this is available, that the mechanism of phenylhydrazine poisoning is similar in all the tissues studied,

and that the inhibition of the Pasteur reaction by phenylhydrazine in these tissues is bound up with the inhibition of glucose oxidation. The question at once arises as to whether the continuance of the Pasteur reaction is necessarily linked with oxidation of carbohydrate, or whether the oxidation of other foodstuffs may also bring about the disappearance of glycolysis. This question cannot be regarded as settled at the present time. There are very few cases where the relative amount of carbohydrate oxidised can be assessed with anything approaching the certainty with which an estimate can be formed for tissues such as brain and retina whose continued respiration depends on such supplies. Moreover, peripheral frog-nerve [Gerard, 1932] provides an example where the existence of such a relationship seems doubtful on the basis of existing information; it should be remembered however that this tissue presents metabolic peculiarities, such as the delayed appearance of glycolysis in nitrogen [Gerard and Meyerhof, 1927] not found in other cases [see also Chang and Gerard, 1933]. Again, with sugars other than glucose (*e.g.* fructose), oxidation of carbohydrate may proceed without anaerobic lactic acid formation (brain, see Loebel [1925], brain and retina, see Dickens and Greville [1933, 1]); it cannot however be assumed that oxidation of glucose and fructose by tissues necessarily proceeds along similar routes, and it is therefore permissible to limit the present discussion to the rôle of the oxidation of glucose in the Pasteur mechanism.

As far as the glucose metabolism of the mammalian tissues here described is concerned, the evidence for the hypothesis advanced by Dickens and Šimer [1930, 2], that the Meyerhof cycle is connected mainly or wholly with the oxidation of carbohydrate, rests principally on observations of R.Q. [Dickens and Šimer, 1930, 2; 1931, 2; Dickens and Greville, 1933, 1, 3], on the effect of sugar-lack on the ammonia formation [Dickens and Greville, 1933, 2] and on the respiration and R.Q. [Dickens and Greville, 1933, 1].

If the estimate of sugar oxidation derived from these values be accepted, it is found that in all the normal tissues studied glucose oxidation runs parallel with their ability to form lactic acid from glucose. The criticism which may be applied to this conclusion is that the evidence of carbohydrate oxidation is necessarily indirect in most cases. Though there does not appear to be any reason to doubt the correctness of the estimates of carbohydrate oxidation, some more direct demonstration of a link between carbohydrate oxidation and cleavage is certainly desirable. Phenylhydrazine may be regarded as helping to fill this gap, but the question cannot yet be regarded as answered. Do other inhibitors of the Pasteur reaction behave towards carbohydrate oxidation similarly to phenylhydrazine, or is the inhibition of carbohydrate oxidation merely an incidental property possessed by phenylhydrazine? Unfortunately Warburg's [1926] experience with ethyl *iso*cyanide does not help us to decide this question, since he did not make use of any tissue in which the respiration falls appreciably in absence of glucose in short experiments.

With yeast cells Warburg states that "similar though not equally convincing experiments can be carried out with yeast cells.... Without the respiration of the yeast being obstructed to any considerable extent the aerobic fermentation is intensified in the presence of 10^{-3} *N* ethyl *iso*cyanide. The aerobic fermentation by yeast in 10^{-3} *N* ethyl *iso*cyanide does not however attain the dimension of the anaerobic fermentation." As this experiment would provide a crucial test of the interdependence or otherwise of the fermentation and carbohydrate oxidation in this case, it is proposed to repeat the observations made with phenylhydrazine using ethyl *iso*cyanide and other similar reagents in this and related examples when opportunity occurs.

SUMMARY.

The action of phenylhydrazine and some allied substances on the metabolism of surviving tissues has been investigated. Phenylhydrazine, in low concentration, is without action on the anaerobic glycolysis of animal tissues or on the anaerobic fermentation of yeast. On the other hand, in the presence of oxygen, it causes the aerobic lactic acid formation of a wide range of animal tissues and the aerobic fermentation of baker's yeast to rise to a value near or equal to that of the same cells anaerobically. The effect of the same treatment on the respiration is described, and it is found that the tissues which are dependent on the oxidation of glucose (or lactate) for their continued respiration undergo a progressive fall of respiration after treatment. Baker's yeast also belongs to this class of cell. However, the majority of animal tissues are not so dependent, and their respiration is much less obviously affected by phenylhydrazine. Tumour tissue (Jensen sarcoma) behaves similarly to this group of normal cells, and its already high aerobic glycolysis is caused by this treatment to rise to the anaerobic value. The bearing of these observations on the nature of the Pasteur reaction is discussed.

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LXXVIII. THE HYDROLYSIS OF LECITHO-VITELLIN BY PEPSIN AND BY TRYPSIN-KINASE.

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It has long been recognised that egg-yolk and milk contain somewhat similar proteins. In particular the two phosphoproteins, caseinogen and lecitho-vitellin, show a certain similarity in constitution and properties, though the latter is peculiar in respect of its content of iron and copper. For example Rimington and Kay [1926] found that hydrolysis of caseinogen with 1 % sodium hydroxide liberated all the organically bound phosphorus of the protein as inorganic phosphate. McFarlane [1932], working with lecitho-vitellin, showed that the action of 1 % alkali resulted in the formation of an acid-insoluble material containing 50 % of the original nitrogen, only a trace of phosphorus and no iron, while the acid-soluble products were rich in both iron and phosphorus.

Little information is available with regard to the action of enzymes on lecitho-vitellin. Miescher [1870] observed that an insoluble material separated out from a peptic digest of egg-yolk. This material was named "haematogen" by Bunge [1885], who found that it contained iron in organic combination. The nature of this insoluble product was studied in greater detail by Hugounenq and Morel [1905, 1, 2; 1906] and later by Posternak [1927], whose results indicated that the so-called "haematogen" might not be a pure compound. These investigations were however carried out before the separate preparation of livetin, the second protein of egg-yolk, by Kay and Marshall [1928; see also Plimmer 1908].

The enzymic cleavage of caseinogen has, on the other hand, been extensively investigated. The studies of Rimington [1927, 1, 2], Rimington and Kay [1926] and Holter *et al.* [1932], on the products of enzymic hydrolysis of caseinogen, have thrown considerable light on the structure of the caseinogen molecule. The nature of the enzymic hydrolysis has been further studied by Stirling and Wishart [1932], who observed certain well-defined differences in the digestion of caseinogen by pepsin and by trypsin-kinase. Briefly summarised these differences were as follows:

1. In tryptic digests acid-soluble phosphorus was liberated more rapidly than acid-soluble nitrogen; the reverse held for pepsin.

2. In peptic digests a stage was reached early in digestion (presumably by the liberation of an acid-soluble material containing no phosphorus) in which the residual acid-insoluble material had a N/P ratio of 12·8. Further, the acid-insoluble residue preserved approximately this ratio throughout a prolonged

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period even though it was considerably diminished in amount by further hydrolysis. In the later stages of digestion, however, the N/P ratio fell slowly as a result of the liberation of a nitrogen-rich material. It was suggested that the final acid-insoluble residue might be identical with Linderstrøm-Lang's [Holter *et al.*, 1932] "paranuclein," with a N/P ratio of 8.

3. With trypsin the course of the digestion was entirely different. There was a rapid preferential liberation of acid-soluble phosphorus, so that the N/P ratio of the insoluble residue rose progressively until, under suitable conditions, it reached infinity. At this stage 30 % of the nitrogen still remained acid-insoluble.

On the basis of these findings it was suggested by Stirling and Wishart that trypsin breaks down peptide linkages in proximity to the phosphorus atoms, the resultant phosphorus-containing cleavage products being of small molecular size. On the other hand, the initial peptic rupture occurs at linkages remote from the phosphorus atoms, the phosphorus-containing cleavage products being of much greater molecular dimensions and therefore insoluble in trichloroacetic acid.

It appeared from the results of these experiments that the same method of attack might be usefully employed in elucidating the structure of lecitho-vitellin.

Experimental.

Preparation of lecitho-vitellin.

The intact yolks of 24 eggs were washed in running water and finally in 0·9 % NaCl until free from egg-white. The yolk membrane was punctured and the contents, without membrane and chalazae, mixed with an equal volume of 10 % NaCl and extracted with an equal volume of ether in a mechanical shaker. Extraction was repeated until the ether was colourless and no qualitative test for lecithin could be obtained. Usually 6 or 7 thorough extractions were required, each with an amount of ether equal to the volume of the egg-yolk solution. The saline solution was poured into 20 volumes of distilled water and, after standing overnight, the supernatant liquid was siphoned off from the white protein precipitate. The precipitate was centrifuged and dissolved in 10 % NaCl; the solution was then filtered and poured into 20 volumes of water. The dissolution and precipitation were repeated, the precipitate being washed well with water before centrifuging for the last time. The protein was extracted with cold 80 % and then 97 % ethyl alcohol for 3 hours each and the residue filtered off, washed with absolute alcohol, ether and finally toluene. The resulting protein was easily friable and passed through a 60-mesh sieve.

Two such preparations of lecitho-vitellin were made, identical procedures being followed. The method given differs in two respects from those recorded in the literature: (1) the use of cold ethyl alcohol in the final lipoid extraction; and (2) the addition of toluene after ether as a dehydrating agent (recommended by Van Slyke [1923] for caseinogen). The two products were alike in appearance and general behaviour. Analysis gave the following results:

		I %	II %
	Moisture	22·42	23·9
% dry weight	Nitrogen	15·78	16·78
	Phosphorus	0·83	1·03
	Ash	2·20	2·38

These show very fair agreement with the analyses of Osborne and Campbell [1900] and Calvery and White [1930].

It is stated that pure vitellin is insoluble in water and almost insoluble in dilute alkali; vitellin in loose combination with lecithin is relatively soluble. Both the above preparations were soluble up to 1 % in approximately $M/40$ KOH. Though insoluble directly in acid, an acid solution could readily be obtained by the addition of acid to the original alkaline solution. The solubility of the preparations at first suggested that they still contained considerable quantities of lecithin, but extraction of the protein with boiling ethyl alcohol showed that the lecithin content could not be more than 2 %.

Experiments were begun with preparation I but unfortunately this did not suffice for all the experiments, and for some of the later experiments the second preparation had to be used. A determination of the N/P ratio of the substrate solution was however made during each experiment, and these figures are recorded in the tables. In evaluating the changes of N/P ratio which occur during the course of each digestion, due consideration must be given to the initial value of this ratio.

Vitellin solutions. In making solutions of the protein for the enzymic hydrolyses we adopted the following method. A weighed quantity (about 2 g.) of vitellin was rubbed up to a smooth paste with a little water, further water was then added to a total volume of about 50 ml.; 2·5 ml. M KOH per g. of vitellin were then added slowly and the rubbing up continued until the preparation, which first became viscous, again liquefied. The total volume was now made up to 75 ml. per g. of vitellin and the solution centrifuged after standing overnight. The supernatant liquid was used for the digestion experiment. The solution so obtained had p_H approximately 9 and, when added to the buffer of p_H 8·8 for the trypsin experiments, did not materially alter the p_H of the latter. For the pepsin experiments a predetermined quantity of N HCl was added to the vitellin solution to bring the p_H approximately to 2 before mixing with the acid buffer. The vitellin, at first precipitated by this acid, redissolved immediately on further addition of acid. Determination of the p_H at various stages during the enzymic hydrolyses showed that it remained well within the optimum range for each enzyme.

By this method we were never able to prepare solutions of higher concentration than about 1 %. This was unfortunate since the nitrogen and phosphorus contents of the enzyme and substrate controls then formed not inconsiderable proportions of the total nitrogen and total phosphorus present. In all digestions substrate and enzyme controls were followed throughout the time course of the experiment, and the tabulated values are net values.

Enzyme solutions. Solutions of B.D.H. trypsin and B.D.H. pepsin were used. The concentration of 0·75 % was chosen after carrying out 2 experiments, one with trypsin and one with pepsin, in which a series of different concentrations of enzyme was allowed to act on the substrate for a period of 2 hours. The results of these experiments are reported in Tables III A and III B.

Buffer solutions. The buffer solutions consisted of a KCl-borate mixture of p_H 8·8 and a KCl-HCl mixture of p_H 1·8. On occasion these were employed in a concentration 4 times that given in the previous paper in order that the volume saved thereby might be utilised for additional substrate solution (see below).

Procedure.

This was identical with that described in the previous paper [Stirling and Wishart, 1932] except that the digest-tubes on occasion contained 8 ml. of substrate and 1 ml. of the more concentrated buffer instead of 5 ml. and 4 ml. respectively. A large number of experiments were carried out in which the time-

Table IA. *Trypsin-kinase (7·5 mg. in 10 ml. solution).*

Exp. no.	5	6
Total N (mg. per 10 ml. sol.)	11·61	5·68
Total P (mg. per 10 ml. sol.)	0·538	0·348

	Exp. 5				Exp. 6			
	Acid-sol. N		Acid-sol. P		Acid-sol. N		Acid-sol. P	
Time	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total
10 mins.	0·53	4·6	0·059	11·0	0·43	7·6	0·055	15·8
20 mins.	0·97	8·3	0·095	17·7	0·69	12·1	0·084	24·1
30 mins.	1·35	11·6	0·119	22·1	0·88	15·5	0·097	27·8
40 mins.	1·58	13·6	0·126	23·4	1·13	20·0	0·111	31·8
50 mins.	1·98	17·0	0·149	27·6	1·32	23·2	0·114	32·7
60 mins.	2·17	18·7	0·173	32·1	1·50	26·4	0·093	26·6
2 hrs.	3·14	27·0	0·221	41·0	1·95	34·2	0·150	43·0
3 hrs.	3·91	33·7	0·238	44·2	2·27	39·9	0·162	46·4
4 hrs.	4·54	39·1	—	—	2·65	46·6	0·188	54·0
5 hrs.	5·08	43·8	0·255	47·2	2·73	48·0	0·187	53·6
6 hrs.	5·36	46·2	0·268	49·9	2·98	52·5	0·191	54·7
9 hrs.	6·72	57·9	0·306	57·0	3·19	56·2	0·216	62·0
12 hrs.	7·13	61·4	0·335	62·0	3·60	63·3	0·214	61·2
24 hrs.	8·39	72·3	0·376	70·0	4·62	81·3	0·239	68·5

Table IB. *Pepsin (7·5 mg. per 10 ml. solution).*

Exp. no.	8	9
Total N (mg. per 10 ml. sol.)	5·31	9·82
Total P (mg. per 10 ml. sol.)	0·237	0·460

	Exp. 8				Exp. 9			
	Acid-sol. N		Acid-sol. P		Acid-sol. N		Acid-sol. P	
Time	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total	Amount mg.	% of total
10 mins.	1·89	35·6	0·039	16·4	2·46	25·0	—	—
20 mins.	2·63	49·6	0·058	24·4	3·55	36·1	0·072	15·7
30 mins.	2·88	54·4	0·069	29·0	4·35	44·3	0·087	18·9
40 mins.	3·02	57·0	0·074	31·1	4·78	48·7	0·016	23·0
50 mins.	3·26	61·5	0·076	31·9	5·17	52·7	0·105	22·8
60 mins.	3·37	63·6	0·072	30·3	5·44	55·4	0·113	24·6
2 hrs.	3·44	64·9	0·065	27·3	6·28	63·9	0·123	26·7
3 hrs.	3·86	72·8	0·058	24·4	6·50	66·2	0·128	27·8
4 hrs.	3·99	75·2	0·083	34·9	6·84	69·6	0·129	28·0
5 hrs.	4·12	77·6	0·071	29·8	6·94	70·6	0·133	28·9
6 hrs.	4·21	79·4	0·063	26·5	7·15	72·8	0·131	28·5
9 hrs.	4·21	79·4	0·063	26·5	7·41	75·5	0·129	28·0
*12 hrs.	4·20	79·2	0·050	21·0	7·64	77·8	0·127	27·6
24 hrs.	4·40	82·8	0·044	18·5	6·83	69·5	0·124	27·0

* 15 hrs. in exp. no. 8.

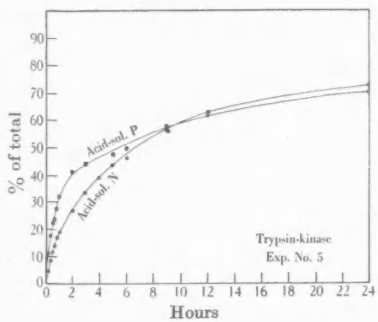


Fig. 1.

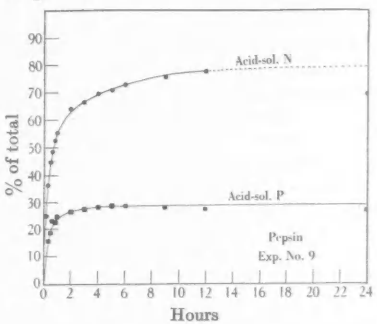


Fig. 2.

course of the digestion of various concentrations of substrate by 0·75 % enzyme was followed. Of these, 2 experiments for each enzyme are given in Tables I A (trypsin) and I B (pepsin). Graphs are also given for 1 experiment with each enzyme (Figs. 1 and 2). The nitrogen-phosphorus ratios of the acid-soluble and acid-insoluble material at each stage in these experiments are given in Tables II A and II B.

Table II A. *N : P ratios.*

Exp. no.			5	6
N : P ratio of original substrate			21·6	16·4
	Exp. 5		Exp. 6	
Time	Acid-sol.	Acid-insol.	Acid-sol.	Acid-insol.
10 mins.	9·1	23·2	7·9	18·0
20 mins.	10·2	24·1	8·1	19·0
30 mins.	11·3	24·6	9·1	19·2
40 mins.	12·6	24·4	10·2	19·2
50 mins.	13·2	24·8	11·5	18·7
60 mins.	12·6	25·8	16·1	16·4
2 hrs.	14·2	26·7	13·0	18·9
3 hrs.	16·5	25·7	14·0	18·4
4 hrs.	—	—	14·1	18·9
5 hrs.	19·9	23·1	14·6	18·4
6 hrs.	20·0	23·2	15·6	17·2
9 hrs.	22·0	21·1	14·8	18·8
12 hrs.	21·2	22·0	16·8	15·6
24 hrs.	22·1	19·9	19·4	9·8

Table II B. *N : P ratios.*

Exp. no.			8	9
N : P ratio of original substrate			22·4	21·5
	Exp. 8		Exp. 9	
Time	Acid-sol.	Acid-insol.	Acid-sol.	Acid-insol.
10 mins.	48·6	17·1	—	—
20 mins.	45·5	15·0	49·4	16·1
30 mins.	41·7	14·4	50·0	14·6
40 mins.	40·9	14·0	45·1	14·2
50 mins.	43·1	12·7	49·3	13·1
60 mins.	46·8	11·7	48·3	12·6
2 hrs.	52·8	10·8	51·0	10·5
3 hrs.	66·6	8·1	50·9	10·0
4 hrs.	48·0	8·5	52·9	9·0
5 hrs.	58·0	7·4	52·2	8·8
6 hrs.	66·8	6·3	54·5	8·1
9 hrs.	66·8	6·3	57·5	7·3
*12 hrs.	84·0	5·9	60·2	6·5

* 15 hrs. in exp. no. 8.

Tables III A and III B show the results obtained when the same substrate solution was digested for a uniform period of 2 hours with enzyme concentrations varying from 100 mg. to 1 mg. per 10 ml.

In addition to these, a third type of experiment was carried out which has no parallel in the previous paper. The production of an insoluble precipitate, analogous to that named "haematogen," by Bunge, was a striking feature of the peptic digests. We therefore carried out a series of digestions with enzyme concentrations of 100 mg. and 20 mg. of pepsin per 10 ml. in which no trichloroacetic acid was added, but the insoluble material was quickly filtered off, and the nitrogen and phosphorus contents of the filtrate were examined. Table IV A records the results of this experiment and shows the early formation of this "haematogen" material and its remarkable resistance to peptic hydrolysis. Table IV B records the nitrogen-phosphorus ratios for filtrate and residue in this experiment.

Table III A. *Effect of varying trypsin concentration.*

Total N 8·95 mg. per 10 ml.
Total P 0·579 mg. per 10 ml.
N : P ratio of original substrate 15·5
Time of incubation 2 hours.

Trypsin (mg. per 10 ml. sol.)	Acid-sol. N as % of total N	Acid-sol. P as % of total P	N : P ratio	
			Acid-sol. material	Acid-insol. material
50	82·5	61·8	20·6	7·1
25	74·5	58·2	19·8	9·4
10	56·7	40·6	21·6	11·3
5	40·7	38·2	16·5	14·8
2·5	27·2	34·5	14·4	15·9
1	15·1	19·3	12·0	16·3

Table III B. *Effect of varying pepsin concentration.*

Total N 11·10 mg. per 10 ml.
Total P 0·577 mg. per 10 ml.
N : P ratio of original substrate 19·2
Time of incubation 2 hours.

Pepsin (mg. per 10 ml. sol.)	Acid-sol. N as % of total N	Acid-sol. P as % of total P	N : P ratio	
			Acid-sol. material	Acid-insol. material
100	77·8	38·1	39·3	6·9
50	77·2	32·2	46·1	6·5
25	74·7	30·7	46·7	7·0
10	69·7	30·1	44·5	8·3
5	61·3	29·2	40·3	10·5
1	34·3	15·9	41·4	15·0

Table IV A.

Exp. no.	10	11
Total N (mg. per 10 ml.)	8·08	8·08
Total P (mg. per 10 ml.)	0·501	0·501
Enzyme conc.	100 mg.	20 mg.

Time	Composition of filtrate			
	% N	% P	% N	% P
1 hr.	86·5	25·5	80·0	19·9
2 hrs.	86·1	27·5	79·3	22·4
3 hrs.	—	—	78·5	22·8
4 hrs.	—	—	79·8	22·8
9 hrs.	—	—	82·7	25·3
24 hrs.	—	—	87·8	27·2

Table IV B. *N : P ratios of filtrate and residue.*

Exp. no.	10	11
N : P ratio of original substrate	16·1	16·1
Enzyme conc.	100 mg.	20 mg.

Time (hrs.)	N : P ratio of			
	Filtrate	Residue	Filtrate	Residue
1	54·5	2·9	64·7	4·0
2	50·3	3·1	57·1	4·3
3	—	—	55·5	4·5
4	—	—	56·3	4·25
9	—	—	52·6	3·7
24	—	—	52·1	2·7

Discussion.

There are certain points of resemblance between the experiments now reported and those recorded by Stirling and Wishart [1932], but the resemblance is not complete—a circumstance which may be ascribed to the apparently greater complexity of the lecitho-vitellin molecule.

As with caseinogen, the first step in the degradation of the protein by either enzyme does not yield products which are completely soluble in trichloroacetic acid. This is evident from the N/P ratios of the earlier stages in Tables II A and II B, and it confirms the criticism, made in the previous paper, concerning experiments on the kinetics of enzyme action which are based on the assumption that trichloroacetic acid precipitates only the unattacked substrate.

As with caseinogen there is a preferential liberation of acid-soluble phosphorus in tryptic digests and of acid-soluble nitrogen in peptic hydrolysis. With trypsin however the resemblance is only superficial. With the lower concentrations of caseinogen substrate, all the phosphorus was rendered acid-soluble, so that the nitrogen-phosphorus ratio of the acid-insoluble material gradually rose to infinity. In the present experiments (Table I A) the preferential liberation of phosphorus by trypsin ceases when about half of the nitrogen and phosphorus have been rendered acid-soluble, and thereafter the nitrogen liberation may even gain on that of the phosphorus. A similar condition is revealed in the results shown in Table III A, where the time of reaction was kept constant, and the enzyme concentration was varied. With the lower enzyme concentrations more phosphorus than nitrogen was rendered acid-soluble; with concentrations which would liberate about 40 % of the total nitrogen the percentage liberations of nitrogen and phosphorus were equal; while, with the stronger enzyme concentrations, there was a relatively greater liberation of acid-soluble nitrogen.

It is also noteworthy that in none of the experiments with lecitho-vitellin, however prolonged the digestion, or however strong the enzyme concentration, have we been able to secure a liberation of more than about 80 % of the total nitrogen and 70 % of the total phosphorus of the substrate.

The results of Stirling and Wishart [1932] on caseinogen were explicable on the assumption that the phosphorus of caseinogen was concentrated in one relatively small group. The results of the time-course experiments on lecitho-vitellin, together with the maxima of hydrolysis, suggest that such a hypothesis will not hold for lecitho-vitellin, and that there must be at least two phosphoric complexes in the molecule, one having similar properties to that of caseinogen and the other being highly resistant to tryptic hydrolysis.

Turning to the peptic digestions, Tables I B and III B show that there is a preferential liberation of acid-soluble nitrogen. In Tables IV A and IV B it may be seen that, with an enzyme concentration of 20 mg., the shorter periods of digestion result in the formation of an insoluble material or "haematogen" embodying about 20 % of the original nitrogen and 77 % of the original phosphorus, thus giving a N/P ratio of just over 4. This "haematogen" is obviously resistant to further peptic hydrolysis since it remains unchanged for at least 4 hours in the presence of the weaker enzyme concentration. Indeed, it is only at the ninth and twenty-fourth hours at this enzyme concentration that further degradation of the "haematogen" is observed. The change is however slight and, as Table IV A shows, affects the nitrogen slightly more than the phosphorus. With the stronger enzyme concentration degradation is more rapid.

Turning now to Exps. 8 and 9 (Tables I B and II B), in which the cleavage products were separated by trichloroacetic acid, it appears that "haematogen"

is acid-insoluble—a fact which we have confirmed directly—and that digestion is confined mainly to the non-haematogen portion, proceeding towards the attainment of a N/P ratio in the acid-insoluble material approximating to that of the insoluble material of Tables IV A and B. (The ratios are not identical, but due weight must be given to the fact that in Table IV B the N/P ratio of the original substrate is 16, while in Table II B, it approximates to 21.)

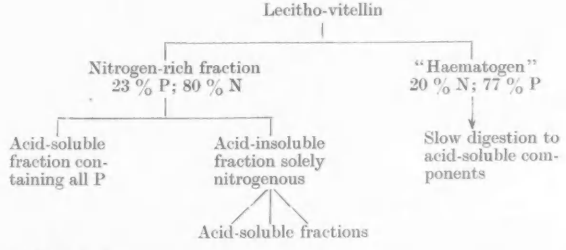
In the digestion of the non-haematogen portion the liberation of the phosphorus of this fraction in acid-soluble form is more rapid than that of the nitrogen of the same fraction. This can be brought out by expressing the amounts of acid-soluble nitrogen and phosphorus, say in Exp. 9 (Table I B), not as percentages of the total nitrogen and phosphorus, but as percentages of the non-haematogen nitrogen and phosphorus, these being taken as 7·64 mg. and 0·13 mg. respectively. The results are as follows:

Experiment No. 9. To show relative rates of liberation of phosphorus and nitrogen from the non-haematogen portion.

Total non-haematogen-nitrogen assumed to be 7·64 mg.
Total non-haematogen-phosphorus assumed to be 0·130 mg.

Time	Acid-sol. N as % of total non-haematogen-N	Acid-sol. P as % of total non-haematogen-P
10 mins.	32·2	—
20 mins.	46·4	55·5
30 mins.	56·8	67·0
40 mins.	62·6	81·6
50 mins.	67·6	80·8
60 mins.	71·1	87·0
2 hrs.	82·0	94·8
3 hrs.	85·1	98·5
4 hrs.	89·8	99·1
5 hrs.	91·0	104·0
6 hrs.	93·6	101·0
9 hrs.	97·0	—
12 hrs.	100·0	—
24 hrs.	89·2	—

These observations seem to allow us to envisage at least 4 stages in the peptic digestion of lecitho-vitellin, which, although occurring for the most part simultaneously, may be thus separately described. First, a cleavage of the protein molecule into 2 fractions, one containing 80 % of the original nitrogen and 23 % of the phosphorus, and the other being "haematogen." Second, in the nitrogen-rich fraction, disruption of peptide linkages close to its phosphorus group leads to the rapid liberation of all this phosphorus in acid-soluble form, leaving a residue which is entirely nitrogenous and still of sufficient complexity to be acid-insoluble. The third stage is the disruption of this nitrogenous



residue into acid-soluble fractions. Finally there is the very slow and feeble digestion of the "haematogen" portion of which we have but slight evidence. This series of reactions is shown concisely in the diagram (p. 557).

It is evident that such a conception of the process of peptic digestion requires the presence of at least two widely separated or dissimilar phosphoric complexes in the vitellin molecule. This assumption is in accordance with the observations on tryptic digestion already discussed.

In this connection it is interesting to note that in tryptic digestion only 70 % of the protein-phosphorus could be rendered acid-soluble, whilst in peptic digestion a somewhat similar amount (77 %) of the phosphorus remained acid-insoluble as the "haematogen" fraction and was highly resistant to further peptic digestion. One might be tempted to assume identity between these fractions. Such an assumption seems however to be unwarranted in view of Posternak's [1927] preparation from the digestion of the residue from ether extraction of egg-yolk of a fraction rich in both phosphorus and iron, which is highly resistant to both peptic and tryptic actions.

Summary.

The action of trypsin-kinase and pepsin on lecitho-vitellin has been studied. Products are formed which are not completely soluble in trichloroacetic acid. With pepsin the rate of liberation of acid-soluble nitrogen is greater than that of acid-soluble phosphorus, and the production of "haematogen" with a N/P ratio of 4·25 is a striking feature of these digests.

With trypsin the rate of liberation of acid-soluble phosphorus relative to that of acid-soluble nitrogen has been shown to depend on the enzyme concentration employed. The maximum of hydrolysis is reached, however, at all concentrations at 80 % of the total nitrogen and 70 % of the total phosphorus.

It is considered that the vitellin molecule must contain at least two widely separated or dissimilar phosphoric complexes, one being highly resistant to enzymic attack.

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LXXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVII. (*a*) ON THE PRODUCTION OF HYDROXYANTHRAQUINONES BY SPECIES OF *HELMINTHOSPORIUM*. (*b*) ISOLATION OF TRITISPORIN, A NEW METABOLIC PRODUCT OF *HELMINTHOSPORIUM TRITICI-VULGARIS* NISIKADO. (*c*) THE MOLECULAR CONSTITUTION OF CATENARIN.

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IT has been previously shown [Charles *et al.*, 1933] that the mycelium of *Helminthosporium gramineum* Rabenhorst, when grown on a synthetic medium containing glucose as the sole source of carbon, contains surprisingly large amounts (30 %) of hydroxyanthraquinones, consisting of a mixture of helminthosporin, $C_{15}H_{10}O_5$ (I), and hydroxy*iso*helminthosporin, $C_{15}H_{10}O_6$ (III), approximately in the proportion of two to three parts of the former to one part of the latter. Further, we have shown [1933, 1] that the mycelium of *H. cynodontis* Marignoni and *H. euchlaenae* Zimmermann, when grown under the same conditions, contains in each case a third new hydroxyanthraquinone, cynodontin, $C_{15}H_{10}O_6$ (II). This hydroxyanthraquinone is the only one produced by *H. euchlaenae*, but it is accompanied in the case of *H. cynodontis* by small amounts of helminthosporin (I).

These observations have now been extended to many different species and strains of *Helminthosporium*, about 40 in all, most of which were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland. While this further work has resulted in the discovery of only one hitherto undescribed hydroxyanthraquinone, it has led to a number of interesting observations which are recorded in this paper; numbers of the species examined gave no trace of hydroxyanthraquinones, but some of them gave considerable yields of one or more of the above-mentioned hydroxyanthraquinones.

The procedure followed was to grow the chosen species on a Czapek-Dox solution, filter off and dry the mycelium and extract it with a suitable solvent, *e.g.* chloroform or acetone. Under these conditions the results described have been obtained with the following species:

(*a*) *H. catenarium* Drechsler. Considerable yields of hydroxy*iso*helminthosporin (III) in an almost pure condition were isolated, and hence it is proposed to change the name of this hydroxyanthraquinone from that previously given

it to *catenarin* (III). *H. catenarium* was originally described by Drechsler [1923], who isolated it from wood reed grass (*Cinna arundinacea* L.) occurring near Brookline, New York, on the northern coast of Long Island. Drechsler gave the name *H. catenarium* to the species "because of its tendency toward the formation of chains of spores in nature as well as in pure culture."

(*b*) *H. tritici-vulgaris* Nisikado. Considerable yields of catenarin (III), together with smaller amounts of the new metabolic product *tritisporin* (see below) and helminthosporin (I) were isolated. Nisikado [1929, 1, 2] isolated and described *H. tritici-vulgaris* from diseased wheat (*Triticum vulgare* Vill.). This organism produces on wheat leaves a yellow spot disease which is of fairly common occurrence in Japan.

(*c*) *H. velutinum* Link. The only hydroxyanthraquinone isolated from this species was catenarin (III), but the mycelium also contained appreciable amounts of ergosterol. *H. velutinum* Link was first described by Link as long ago as 1809, and was found by him to be parasitic upon beech wood.

(*d*) *H. avenae* Eidam. Cynodontin (II) was isolated in small amounts and in almost pure condition. It was accompanied by small amounts of ergosterol. *H. avenae* was described originally by Eidam [1891], who found this organism on, and causing leaf spot disease of, oats occurring in Silesia. The disease on oats caused by *H. avenae* is widely distributed, having been recorded from almost all parts of the world.

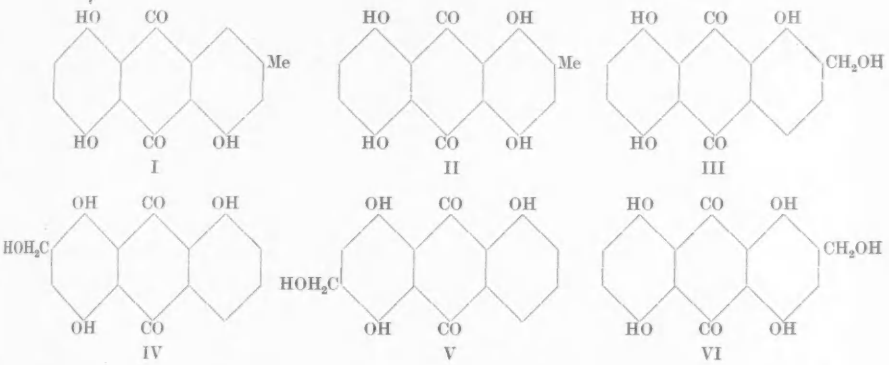
Definite evidence was obtained by colour reactions of the production of hydroxyanthraquinones, although in amounts too small for isolation and identification, by the following species of *Helminthosporium*, which are arranged approximately in the order of decreasing intensity of the colour reactions given: *H. brizae* Nisikado, *H. pedicellatum* Henry, *H. geniculatum* Tracy and Earle, *H. leptochloae* Nisikado and Mke, *H. teres* Sacc., *H. filicicola* P. Henn, *H. torulosum* (Syd.) Ashby, *H. coicis* Nisikado, *H. gibberosporum* Curzi and *H. Yamadai* Nisikado.

No hydroxyanthraquinones could be detected by colour reactions from any of the following species: *H. setariae* Sawada, *H. interseminatum* Berk. and Rav., *H. anomalum* Abbott, *H. monoceras* Drechsler, *H. sacchari* Butler, *H. sativum* P.K. and B., *H. nodulosum* (B. and C.) Sacc., *H. hurae* P. Henn, *H. oryzae* Br. de Haan, *H. turcicum* Passer, *H. Kusanoi* Nisikado, *H. Leersii* Atkinson, *H. maydis* Nisikado and Mke, *H. panici-miliacei* Nisikado, *H. Ravenelii* Curtis and *H. zizaniae* Nisikado.

The results obtained point to an interesting correlation between the biochemical synthesis of hydroxyanthraquinones by species of *Helminthosporium* and their morphological characteristics. Nisikado [1929, 2] divides the genus *Helminthosporium* into two sub-genera on morphological grounds, (1) sub-genus *Eu-Helminthosporium*, (2) sub-genus *Cylindro-Helminthosporium*. Although Nisikado did not examine all the species which we have worked upon, and hence any generalisation may be somewhat inaccurate, it is interesting to find that four of those seven species of *Helminthosporium* which we have found to give good yields of hydroxyanthraquinones, *i.e.*, *H. gramineum*, *H. catenarium*, *H. tritici-vulgaris* and *H. avenae*, are placed by Nisikado in the sub-genus *Cylindro-Helminthosporium*. The other three species, *H. cynodontis*, *H. euchlaenae* and *H. velutinum*, were not examined by Nisikado. On the other hand, twelve of the sixteen species giving entirely negative results, and four of the ten species which only give traces of hydroxyanthraquinones are placed by Nisikado in the sub-genus *Eu-Helminthosporium*. The remaining species were not examined by Nisikado with the exception of *H. teres* Sacc. which is placed by him in the

sub-genus *Cylindro-Helminthosporium*, and which we found to give only traces of hydroxyanthraquinones.

The molecular constitutions of helminthosporin (I) and of cynodontin (II) have already been satisfactorily elucidated [1933, 1, 2], but that of catenarin (III), formerly known as hydroxy*iso*helminthosporin, was left unsettled by previous work. Evidence is now presented, showing that catenarin must be a β-hydroxymethyl-1:4:5-trihydroxyanthraquinone having one of the formulae (III), (IV) or (V). The evidence for this is briefly as follows.



Distillation of catenarin with zinc dust yields β-methylanthracene in rather small quantities—relatively less than helminthosporin. As has been previously reported [Charles *et al.*, 1933], all efforts to oxidise the side-chain in catenarin acetate to carboxyl failed; in every case most of the starting material was recovered unchanged, the rest being completely destroyed. The oxidation of catenarin with concentrated nitric acid was also studied, but apart from a minute quantity of a brownish-yellow substance—apparently a tetranitrocatenarin—the only solid product which could be isolated was oxalic acid. The behaviour of catenarin towards such oxidising agents as nitric acid and chromic anhydride is strongly reminiscent of that of morindone [Simonsen, 1918], which has been shown by synthesis [Jacobson and Adams, 1925; Bhattacharya and Simonsen, 1927] to be 1:5:6-trihydroxy-2-methylanthraquinone. Similarly, the methyl group in cynodontin acetate resists oxidation [Raistrick *et al.*, 1933, 1]. It is therefore reasonable to assume that catenarin contains a nuclear hydroxyl adjacent to a β-methyl or β-hydroxymethyl group. Further evidence for the structure of catenarin may be stated briefly as follows:

(1) Catenarin is unaffected by heating for 8 hours at 100° with methyl iodide and sodium methoxide. Thus, despite its solubility in aqueous sodium carbonate, catenarin appears to contain no β-hydroxyl groups, as these are methylated under such conditions.

(2) Some support for the view that catenarin contains a hydroxymethyl group is afforded by the fact that its acetate, on heating with a saturated solution of hydrogen bromide in acetic acid, yields a product containing bromine. As this product is not homogeneous and the bromine content is low, this evidence is inconclusive.

(3) On oxidation with manganese dioxide and concentrated sulphuric acid, catenarin gives a hydroxycatenarin (VI), $C_{15}H_{10}O_7$, which is but a feeble mordant

dyestuff. Any anthraquinone derivative containing five nuclear hydroxyls is of necessity a derivative of alizarin and it follows that hydroxycatenarin can contain only four, and catenarin, consequently, only three, nuclear hydroxyl groups. The colour of its solution in concentrated sulphuric acid—blue, with a red fluorescence—taken in conjunction with its feeble dyeing properties, indicate that hydroxycatenarin is probably a derivative of 1:4:5:8-tetrahydroxyanthraquinone. Its production from catenarin in the manner described limits the possible formulae for the latter substance to the three expressions III, IV and V.

(4) That catenarin, like helminthosporin and cynodontin, contains nuclear hydroxyls in the 1 : 4 : 5-positions, is further borne out by the similarity of the absorption curves of the three substances in concentrated sulphuric acid solution (see Fig. 1).

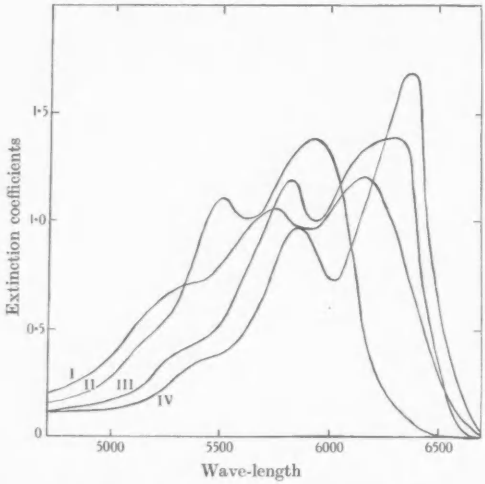


Fig. 1. I. Catenarin. II. Helminthosporin. III. Cynodontin. IV. Hydroxycatenarin.

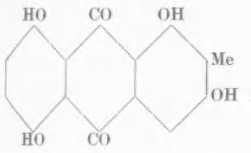
Constitution of tritisporin. This substance, of which only a small amount has been available for investigation, appears to have the formula $C_{15}H_{10}O_7$; it has been analysed as its pyridine salt and as a penta-acetyl derivative. It is only a weak adjective dyestuff and cannot be an alizarin derivative. Furthermore, although readily soluble in aqueous sodium carbonate, its acetate is insoluble in alkaline solutions. Hence the substance does not contain a carboxyl group. On distillation with zinc dust it affords β-methylanthracene.

The colour reactions and fluorescent properties are generally similar to those of catenarin and we are of the opinion that tritisporin must be a β-(hydroxymethyl)-derivative of 1:3:5:7-, 1:3:6:8-, 1:4:5:8- or 1:3:5:8-tetrahydroxyanthraquinone. The 1:4:5:8-orientation is at once excluded because this arrangement is required for hydroxycatenarin (VI), which is isomeric with, but very different from, tritisporin.

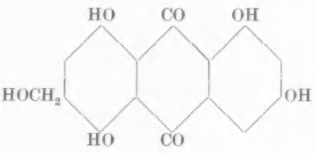
The colour reactions of tritisporin are also very divergent from those of anthrachrysone as described in the literature. For further confirmation we prepared a substance which was undoubtedly 1:3:5:7-tetrahydroxy-2:6-

dimethylanthraquinone (dimethylanthrachrysone) by the action of sulphuric acid at 100° on 2:6-dihydroxy-*p*-toluic acid. The product was sublimed in orange needles and gave a yellow non-fluorescent solution in acetic acid, an orange-yellow solution in aqueous sodium carbonate, a reddish orange solution in aqueous sodium hydroxide and a deep cherry-red solution in sulphuric acid. This behaviour is very unlike that of tritisporin, and it is improbable that the latter can be a derivative of anthrachrysone. It seems that 1:3:6:8-tetrahydroxyanthraquinone is unknown, but it would probably resemble anthrachrysone, much as chrysazin resembles anthrarufin.

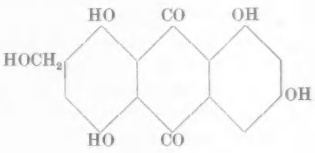
For these reasons we consider that tritisporin is a derivative of 1:3:5:8-tetrahydroxyanthraquinone. It happens that a pure, analysed specimen of 1:3:5:8-tetrahydroxy-2-methylanthraquinone (VII) was available (unpublished synthesis by Dr E. H. Charlesworth) and this was found to be generally



VII



VIII



IX

similar and yet somewhat different from tritisporin in its reactions. In fact the substance VII resembled catenarin more closely in respect of the colour of its solution in sulphuric acid.

All the pigments isolated from species of *Helminthosporium* give fluorescent solutions in acetic acid, a property dependent on the quinizarin grouping. The differences existing between catenarin and VII on the one hand and tritisporin on the other suggest that catenarin should be represented by the formula III and one of the constitutions VIII or IX should be attributed to tritisporin.

Experimental.

A quantity of Czapek-Dox medium of the following composition was made up: glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·01 g.; distilled water, 1000 ml. 350 ml. quantities of this medium were distributed in each of a number of 1-litre conical flasks and sterilised. A measured quantity, previously determined, of sterile *N* sodium hydroxide solution was added to each flask to bring the p_H of the medium to 8·0. Five flasks were then each sown with a suspension in sterile Czapek-Dox medium of a pure culture of the species of *Helminthosporium* under investigation. The inoculated flasks were incubated at 24° for about 7 weeks, filtered, the mycelium dried in a vacuum-oven, ground to a fine powder and exhaustively extracted with chloroform in a Soxhlet apparatus. The chloroform extract was evaporated to dryness and portions of the dry extract were tested with strong sulphuric acid, aqueous sodium hydroxide solution, and concentrated ammonia. As mentioned in the introduction, a large number of species gave either entirely negative colour reactions, or where positive gave such small yields of hydroxyanthraquinones as to render isolation impracticable. In these cases no further investigation was carried out. In a few cases however considerable yields of

hydroxyanthraquinones were produced and these have been more exhaustively studied. The species chosen for further investigation were:

(*a*) *H. gramineum* Rabenhorst.
(*b*) *H. cynodontis* Marignoni.
(*c*) *H. euchlaenae* Zimmermann.
(*d*) *H. catenarium* Drechsler.
(*e*) *H. tritici-vulgaris* Nisikado.
(*f*) *H. velutinum* Link.
(*g*) *H. avenae* Eidam.

The results obtained with species (*a*), (*b*) and (*c*) have already been reported, and those obtained with (*d*), (*e*), (*f*) and (*g*) form the subject of the present communication.

In each case the cultural conditions, methods of separation and isolation of the crude pigment were similar and were as follows: 100 one-litre conical flasks, each containing 350 ml. Czapek-Dox medium, adjusted to p_H 8·0, were sown with the species under investigation and incubated at 24°. At the end of the incubation period the mycelium was filtered off, washed, pressed, dried in a vacuum-oven and ground to a fine powder in a coffee mill. The powdered mycelium was extracted in a large metal Soxhlet apparatus with chloroform until the chloroform extracts were colourless. The extraction usually occupied several days, and a series of solid fractions separating from the chloroform was obtained. These fractions were then examined for their hydroxyanthraquinone content. The final chloroform mother-liquors were evaporated to dryness and treated with light petroleum, by which means a considerable quantity of "fat" was isolated. The experimental details for each of the species examined are as follows.

(A) *Helminthosporium catenarium* Drechsler.

The culture used was purchased from Baarn and had been received by them in 1929 from Nisikado, who isolated it from *Beckmannia*. Incubation period, 65 days. Residual glucose, 0·65 %. The surface of the mycelium was flame-coloured and slightly buckled with a chocolate-brown reverse. Weight of mycelium from 97 flasks, 204 g. The chloroform extract was bright orange in colour and gave the following fractions:

(*a*) Fraction 1. First day's extraction. Weight, 9·96 g.; M.P. 220–226°.
(*b*) Fraction 2. Second and third day's extraction. Weight, 3·72 g.; M.P. 244–245°.
(*c*) Fraction 3. Fourth, fifth and sixth day's extraction. Weight, 6·10 g.; M.P. 193–205°.
(*d*) Fraction 4. *Ex.* mother-liquors from fractions 1, 2 and 3. Weight, 2·35 g.; M.P. 193–198°.

The extraction in the Soxhlet proved very tedious, and hence the mycelium was now re-extracted by boiling with a large volume of chloroform giving:

(*e*) Fraction 5. Weight, 11·29 g.; M.P. 244–247°, and
(*f*) Fraction 6. Weight, 9·30 g.; M.P. 244–246°.

Even now the pigment was not completely extracted, and hence the incompletely extracted mycelium was dried and re-extracted with a large volume of boiling acetone and there were thus obtained:

(*g*) Fraction 7. Weight, 4·72 g.; M.P. 238–241°, and
(*h*) Fraction 8. Weight, 1·55 g.; M.P. 179–181°.

Total weight of crude pigment, 48·99 g.

There were also obtained from the light petroleum extract 16·10 g. of crude "fat."

The colours developed by the various fractions on dissolution in conc. sulphuric acid indicated the probable presence in the crude pigmented material of hydroxy*iso*helminthosporin—henceforth called catenarin, as it is the characteristic product of *H. catenarium*—and helminthosporin. On this basis fractions 1 and 2 consist of catenarin contaminated with a small quantity of helminthosporin; fraction 3 is similar, but contains rather more helminthosporin, whilst fractions 4 and 8 are almost entirely composed of this pigment. Fractions 5 and 6

consist of nearly pure catenarin, and fraction 7 is somewhat similar but contains a trace of helminthosporin. For the isolation of the two pigments in a pure state, fractions 4 and 5 were employed.

Catenarin. The crude material was recrystallised from alcohol, when it formed red, slender plates containing alcohol of crystallisation which it lost slowly in air. It had M.P. 246° and agreed in all its properties with "hydroxy*iso*helminthosporin" (M.P. 246°) from *H. gramineum*; a mixed M.P. with the latter substance showed no depression. (Found in material dried at 110°: C, 63·1; H, 3·7 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.)

Catenarin acetate. The crude pigment was acetylated in the usual way with acetic anhydride containing a trace of concentrated sulphuric acid. After recrystallisation from glacial acetic acid, the acetate was obtained as small lemon-yellow rods, M.P. 234–235° alone or mixed with "hydroxy*iso*helminthosporin" acetate (M.P. 234–235°). (Found: C, 61·1; H, 4·1 %. $C_{23}H_{18}O_{10}$ requires: C, 60·8; H, 4·0 %.)

Isolation of helminthosporin. Helminthosporin acetate. The crude pigment, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid, yielded an acetate, which, after three recrystallisations from glacial acetic acid, formed a mass of fluffy yellow needles, M.P. 225°. (Found: C, 63·6; H, 4·1 %. $C_{21}H_{16}O_8$ requires: C, 63·6; H, 4·0 %.) It had all the properties of helminthosporin acetate, and the mixed M.P. with this substance showed no depression.

Helminthosporin. The above acetate was hydrolysed by warming on the water-bath with 15 % sodium hydroxide during 90 minutes. The deep violet solution was acidified with hydrochloric acid and, after boiling for a few minutes to ensure coagulation, the red-brown precipitate was collected, washed with water, dried and twice recrystallised from pyridine. The product formed flat, maroon-coloured needles with a bronze lustre, M.P. 226–227°. (Found: C, 66·8; H, 3·8 %. $C_{15}H_{10}O_5$ requires: C, 66·7; H, 3·7 %.) It had all the properties of helminthosporin (M.P. 226–227°), and showed no alteration in M.P. when mixed with a sample of the latter substance.

(B) *Helminthosporium tritici-vulgaris* Nisikado.

The culture used was purchased from Baarn and was received by them in November 1929 from Nisikado, strain number 285, who isolated it from diseased wheat, *Triticum vulgare*. Incubation period, 39 days. Residual glucose, 0·88 %. The surface of the mycelium was buff-coloured, somewhat crumpled and with a chocolate-coloured reverse. Weight of mycelium from 100 flasks, 204 g.

The chloroform extract gave the following fractions:

(*a*) Fraction I. Separating from the combined chloroform extracts after 6 days' extraction. Weight, 9·04 g.; M.P. 216–225°.

(*b*) Fraction II. Separating from mother-liquors from fraction I after evaporation to low bulk. Weight, 0·30 g.; M.P. 152–176°.

(*c*) Fraction III. The mother-liquors from fraction II were evaporated to dryness and extracted with light petroleum giving an insoluble residue, fraction III, weight 1·85 g.; M.P. 128–140°.

Total weight of crude pigment, 11·19 g.

There were also obtained from the light petroleum extract 39·3 g. of crude "fat."

Preliminary examination of the colours produced on dissolving samples of the three fractions in concentrated sulphuric acid indicated that fraction I probably consisted largely of catenarin, but the reddish tinge of the solution suggested that some other substance was also present; fractions II and III gave red-violet solutions with weak red fluorescence and appeared to contain some helminthosporin.

The method of isolation of the pigments was the following:

Fraction I was extracted with alcohol in a Soxhlet apparatus for about 3 hours, after which time the liquid siphoning over became distinctly lighter in colour, and the extract was allowed to crystallise. A brownish material (A) was left as unextracted residue. The reddish-brown crystals separating from the alcohol extract were ground and again extracted with alcohol in a Soxhlet, the process being interrupted when a small amount (*ca.* 0·5 g.) remained in the thimble; this residue was combined with (A). The alcohol extract deposited on standing brownish-red plates (B).

Isolation of tritisporin from (*A*). The crude material had an indefinite M.P. 250–260° and contained appreciable quantities of a greyish impurity which was not readily removed by simple recrystallisation. Accordingly the crude substance was acetylated in the usual way with acetic anhydride containing a trace of sulphuric acid, and after several recrystallisations from glacial acetic acid *tritisporin acetate* was obtained as a mass of very fine pale yellow needles, M.P. 215°. (Found: C, 58·5; H, 3·8 %. $C_{25}H_{20}O_{12}$ or $C_{15}H_5O_2(OAc)_5$ requires: C, 58·6; H, 3·9 %.) The substance was insoluble in cold dilute sodium carbonate solution.

This acetate was hydrolysed by heating on the water-bath with aqueous sodium hydroxide (15 %) during 90 minutes. The blue-violet solution was then acidified and boiled for a few minutes to coagulate the reddish-brown precipitate which was collected, washed with water and dried. The product was sparingly soluble in acetic acid and alcohol but the best solvent for recrystallisation was pyridine from which the *tritisporin* separated as long narrow reddish-brown plates with a bronze lustre, apparently as a pyridine salt. (Found: C, 63·6; H, 4·0; N, 3·8 %. $C_{15}H_{10}O_7.C_5H_5N$ requires: C, 63·0; H, 3·9; N, 3·7 %.) Tritisporin separates from alcohol or acetic acid in flat needles slightly browner in colour than the pyridine salt; it has M.P. 260–262° but the M.P. is difficult to determine accurately as considerable sublimation occurs from 250° onwards.

Tritisporin dissolves very readily in cold sodium carbonate solution to a violet-red solution and in caustic soda to a blue-violet solution which is slowly decolorised in the air. Its solution in concentrated sulphuric acid is reddish violet, redder in tone than catenarin solutions, and on addition of boric acid it becomes blue in thin layers and violet in thick layers. Solutions of tritisporin in acetic acid are salmon-pink with a yellowish green fluorescence.

2-Methylanthracene from tritisporin. Tritisporin (120 mg.) was subjected in portions of 20 mg. to distillation with electrolytic zinc dust according to the micro-zinc dust distillation method of Kögl [1924]. The product of each experiment was deposited in the cooler parts of the tube as a mass of yellowish leaflets with a greenish fluorescence. The combined distillates were extracted from the tubes with ether, the solution filtered and the ether removed on the water-bath. The crystalline residue (*ca.* 10 mg.) was now recrystallised from alcohol. After three crystallisations it was obtained as glittering almost colourless leaflets, M.P. 200–201°, which showed all the properties of 2-methylanthracene. The mixed M.P. with a specimen of synthetic 2-methylanthracene (M.P. 203–204°) was 203° while a mixture with anthracene melted at 188–190°.

Isolation of catenarin from (*B*). The crude brownish red material was acetylated in the normal way by heating with acetic anhydride containing a trace of sulphuric acid. After three recrystallisations from acetic acid the acetate was obtained as small lemon-yellow rods, M.P. 234–235° alone or mixed with catenarin acetate.

The above acetate was hydrolysed by heating with aqueous sodium hydroxide (15 %) on the steam-bath during 90 minutes. The deep violet solution was acidified, boiled for a few minutes and the red-brown precipitate collected, washed with water, dried and recrystallised from alcohol. The recrystallised product formed slender red plates containing alcohol of crystallisation and had M.P. 246°. (Found in material dried at 110°: C, 63·0; H, 3·2 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.) It had all the properties of catenarin and the mixed M.P. with a specimen of the latter substance showed no depression.

Preparation of helminthosporin acetate from fractions II and III. Fractions II and III showed a weak red fluorescence when dissolved in concentrated sulphuric acid, and the presence of helminthosporin was suspected. As the quantity present appeared to be small, however, the direct isolation of helminthosporin by crystallisation was deemed impracticable. Accordingly the combined material of the two fractions was extracted with alcohol for a short time to remove any readily soluble impurities and then acetylated in the usual way with acetic anhydride containing sulphuric acid. The yellow acetate was now separated into two portions by fractional crystallisation from acetic acid. The less soluble part, which consisted mainly of catenarin acetate, was discarded. The more soluble fraction which contained the helminthosporin acetate was subjected to repeated crystallisation from acetic acid, and a small amount was finally obtained in pale yellow fluffy needles, M.P. 224–225°, which had all the properties of helminthosporin acetate (M.P. 225°). The mixed M.P. with a sample of helminthosporin acetate was unchanged (a mixture with catenarin acetate melted at *ca.* 200°).

The presence of helminthosporin in the products from *H. tritici-vulgaris* is thus demonstrated.

Owing to unavoidable losses entailed in the separation of the three pigments an accurate analysis cannot be submitted; the coloured material from *H. tritici-vulgaris* is however estimated to consist of 75–80 % catenarin, 20–25 % tritisporin and 2–4 % helminthosporin.

Comparison of colours and fluorescences of solutions of various natural and synthetic pigments of the anthraquinone group in acetic acid.

Quinizarin	Orange-yellow; green fluorescence
Alizarin	Orange-yellow; no fluorescence
Anthrarufin	Yellow; no fluorescence
Chrysazin	Yellow; no fluorescence
1-Hydroxyanthraquinone	Pale yellow; no fluorescence
Hydroxyanthrarufin	Pure yellow; no fluorescence
Purpurin	Deep orange-yellow; weak green fluorescence
Emodin	Greenish yellow; no fluorescence
Morindone	Yellow; no fluorescence
2-Methyl-1:3:5:8-tetrahydroxyanthraquinone	Orange-yellow; strong green fluorescence
Helminthosporin, Catenarin	Brighter orange-yellow, red shade in thin layers; green fluorescence
Tritisporin	Salmon-red; yellowish-green fluorescence
Cynodontin, Hydroxycatenarin	Bluish red, crimson in thin layers; weak greenish yellow fluorescence

It will be observed that fluorescence is characteristic of quinizarin derivatives.

(C) *Helminthosporium velutinum* Link.

The culture used was purchased from Baarn, where it was isolated in July 1929 from *Salix* spec. Incubation period, 83 days. Residual glucose, 0·77 %. This species gave a thick, glutinous orange-red mycelium with an orange-red reverse. Weight of mycelium from 95 flasks, 297 g.

The chloroform extract had an orange-red colour indistinguishable from that given by *H. catenarium*, and gave the following fractions:

(*a*) Fraction 1. Separating from the combined chloroform extracts after 6 days' extraction. Weight, 11·88 g.; M.P. 244–245°.

(*b*) Fraction 2. Separating from mother-liquors from fraction 1 after evaporation to low bulk. Weight, 0·67 g.; M.P. 115–140°.

(*c*) Fraction 3. The mother-liquors from fraction 2 were evaporated to dryness and extracted with light petroleum giving an insoluble residue, fraction 3, weight 2·13 g.; M.P. 126–130°.

Total weight of crude pigment, 14·68 g.

There were also obtained from the light petroleum extract 64·9 g. of crude "fat."

Fractions 1 and 2 of the crude material dissolved in concentrated sulphuric acid to give solutions with the characteristic blue-purple colour shown by catenarin under these conditions. Fraction 3 was lighter in colour than the others, and its sulphuric acid solution was slightly redder and had a weak greenish fluorescence. Fraction 1 furnished, on recrystallisation from alcohol, catenarin, whose identity was established by analysis, M.P. and mixed M.P., and by preparation of its acetate. Fraction 3 contained a colourless substance, which was isolated and shown to be ergosterol (M.P., mixed M.P., colour reactions, and formation of acetate). The remainder of fraction 3, after removal of the ergosterol, consisted essentially of catenarin.

Catenarin. Fraction 1 crystallised from alcohol in red, slender plates containing alcohol of crystallisation, which was lost slowly in air. It had M.P. 246°, alone or mixed with catenarin (M.P. 246°; from *H. catenarium*), and showed all the properties of the latter. (Found in material dried at 110°: C, 63·0; H, 3·8 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.)

The above material, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid, yielded an acetate which crystallised from glacial acetic acid in small, lemon-yellow rods, M.P. 234–235°. The M.P. was not depressed by admixture with catenarin acetate.

Examination of fraction 3. The material (2 g.) was extracted in a Soxhlet with light petroleum (B.P. 40–60°) during several hours.

(A) *Residue.* The residue in the thimble was red in colour, and on heating softened about 200° and melted finally *ca.* 230°. On acetylation, followed by repeated recrystallisation of the product from acetic acid, it gave catenarin acetate, M.P. 234–235°.

(B) *Extract.* The light orange-red light petroleum extract deposited, on cooling, a quantity of faintly pink crystals (0·5 g.). After several recrystallisations from alcohol this product was obtained as colourless, glittering leaflets, M.P. 161–162°. The mixed M.P. with an authentic specimen of ergosterol (M.P. 160–161°) was 161°, and a comparison of the colour reactions (Salkowski, Tortelli-Jaffé, Liebermann-Burchard, Rosenheim) of the two specimens revealed no divergences.

For further proof that the above product was indeed ergosterol, a portion of it (0·1 g.) was acetylated by boiling for one hour with acetic anhydride (0·5 ml.). On cooling, the acetate separated in glistening plates and, after recrystallisation from alcohol, had M.P. 178–179°.

The peculiar colour of the crude fraction 3 in sulphuric acid is explained by the presence of ergosterol. The colour could be artificially produced by adding ergosterol to solutions of catenarin in sulphuric acid.

(D) *Helminthosporium avenae* Eidam.

The culture used was isolated in 1925 by Mr F. T. Brooks, School of Botany, Cambridge, from oats (*Avena sativa* L.) showing signs of leaf blight disease. As the yield of pigment given by this organism was very small, three separate experiments using a total of 252 flasks were carried out. The average incubation period was 12 weeks. The residual glucose varied from 0·05 to 0·40 %. The mycelium of this species was greyish-white on the surface with a red-black reverse. The total weight of mycelium from 252 flasks was 941 g. The pigment was readily extracted with chloroform, and the chloroform extracts, which had a brilliant eosin-red colour reminiscent of cynodontin, were evaporated to moderate bulk and from this there separated:

(*a*) Fraction 1. Weight, 3·60 g.; M.P. about 250°.

(*b*) Fraction 2. The mother-liquors from fraction 1 were evaporated to low bulk, giving fraction 2, weight 1·92 g.; M.P. 245–260°.

(*c*) Fraction 3. The mother-liquors from fraction 2 were evaporated to dryness and extracted with cold light petroleum. The insoluble portion, representing fraction 3, weighed 0·66 g.; M.P. 135–145°.

Total weight of crude pigment from 252 flasks, 6·18 g.

There was also obtained from the light petroleum solution a total weight of crude "fat" of 165·9 g.

Fractions 1 and 2, from their appearance, and the colour of their sulphuric acid solutions—pure blue with a red fluorescence—appeared to consist almost entirely of cynodontin or of hydroxycatenarin. The presence of the latter was however ruled out by the insolubility of the material in cold, aqueous sodium carbonate. Recrystallisation of the crude pigment from pyridine yielded cynodontin, identified by M.P., mixed M.P., and by preparation of its acetate. In order to verify that the colourless fraction 3 was indeed ergosterol, it was further purified and compared with an authentic specimen of the latter compound; its acetate was also prepared.

Cynodontin. The crude material was recrystallised several times from pyridine, when it formed brown leaflets with a fine bronze lustre, M.P. 260°. (Found: C, 62·9; H, 3·4 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.) This product had all the properties of cynodontin, and its M.P. was not depressed on mixing with the latter substance.

The above material, on acetylation with acetic anhydride containing a trace of concentrated sulphuric acid and recrystallisation from glacial acetic acid, gave an acetate forming bright yellow needles or rods, M.P. 224–225° alone or mixed with cynodontin acetate (M.P. 224–225°).

Fraction 3. Ergosterol. Recrystallisation of this, first from light petroleum, then from alcohol, gave colourless, glittering leaflets, M.P. 161–162°, showing no divergence in reactions from a specimen of ergosterol (mixed M.P., colour reactions of Salkowski, Tortelli-Jaffé, Liebermann-Burchard, Rosenheim). On boiling with acetic anhydride for one hour, it gave an acetate crystallising from alcohol in colourless, glistening plates, M.P. 178–179°.

Further experiments with hydroxyisohelminthosporin (catenarin).

It has been found that the M.P. of hydroxy*iso*helminthosporin, previously given as 238° [Charles *et al.*, 1933], can be raised to 246° by conversion into its acetate, followed by hydrolysis with 15 % sodium hydroxide and recrystallisation from alcohol. (Found in material dried at 110°: C, 63·2; H, 3·3 %. $C_{15}H_{10}O_6$ requires: C, 62·9; H, 3·5 %.) As this substance only occurs in very small amount in the products from *H. gramineum*, but is the main coloured product of *H. catenarium*, it has been re-named *catenarin*.

2-Methylanthracene from catenarin. Catenarin (0·5 g.) was distilled with zinc dust at a dull red heat in a current of hydrogen. 2-Methylanthracene was deposited in the cooler part of the tube as colourless leaflets with a green fluorescence; the yield of product was low (total weight from three such experiments, 50 mg.). After recrystallisation from alcohol the substance had M.P. 200° and had all the properties recorded for 2-methylanthracene; a mixture with the latter compound (M.P. 203–204°) had M.P. 202–203°.

Oxidation of catenarin with manganese dioxide and sulphuric acid. Preparation of hydroxycatenarin. Catenarin (2 g.) was dissolved in concentrated sulphuric acid (15 ml.) and finely powdered manganese dioxide (1·5 g.) added gradually with constant stirring, the temperature being maintained at 60–70°. After all had been added, the mixture was kept at 60–70° during a further 15 minutes and then poured into water. A brownish violet precipitate formed, which, on boiling for a short time with water, became dark brown by hydrolysis. The crude product was recrystallised from pyridine and was thus obtained as dark red-brown crystals. For further purification this material was converted into its acetate by boiling for a few minutes with acetic anhydride containing a trace of sulphuric acid. After four recrystallisations from glacial acetic acid hydroxycatenarin acetate was obtained as small greenish-yellow rods, M.P. 233–234° (mixed M.P. with catenarin acetate, *ca.* 210–213°). (Found: C, 58·6; H, 4·0 %. $C_{25}H_{20}O_{12}$ requires: C, 58·6; H, 3·9 %.) Yield, 0·55 g. The above acetate (0·4 g.) was warmed on the water-bath for 90 minutes with 15 % sodium hydroxide solution (30 ml.), when it dissolved slowly to a deep blue solution. After acidifying with hydrochloric acid and boiling for a few minutes to coagulate the dark brown precipitate, the latter was collected and twice recrystallised from pyridine. It separated as small brownish leaflets with a bronze lustre; on heating it underwent partial sublimation above 280° and melted at about 320°. (Found in material dried at 110° in a high vacuum over P_2O_5: C, 59·2; H, 3·5 %. $C_{15}H_{10}O_7$ requires: C, 59·6; H, 3·3 %.)

The crystals separating from pyridine solutions of hydroxycatenarin contain pyridine which is only partly lost on drying at room temperature. In appearance and in many of its properties hydroxycatenarin resembles cynodontin. Thus, it dissolves in concentrated sulphuric acid to a pure blue solution with a red fluorescence, and it possesses very feeble dyeing properties—much feebler than catenarin, although it is admittedly less soluble than the latter substance. This renders it highly probable that hydroxycatenarin is a derivative of 1:4:5:8-tetrahydroxyanthraquinone, while its fifth hydroxyl group must be in the side-chain as it would otherwise be an alizarin derivative and consequently a powerful mordant dyestuff. In view of its properties hydroxycatenarin must be 1:4:5:8-tetrahydroxy-2-(hydroxymethyl)anthraquinone (VI).

Oxidation of catenarin with nitric acid. Catenarin (1·5 g.) was warmed on the water-bath with nitric acid (20 ml. sp. gr. 1·42). After the initial reaction had subsided the mixture was heated on a boiling water-bath till evolution of brown fumes had practically ceased, then evaporated to small bulk and poured into water, a slight brownish precipitate being formed. The mixture was filtered.

Recrystallisation of the solid from acetic acid afforded fine brownish yellow needles (7 mg.); on heating the substance charred at about 250° without melting. It may be a tetranitrocatenarin. (Found: N, 9·4 %. $C_{15}H_6O_{14}N_4$ requires: N, 9·9 %.)

The only solid product which could be isolated from the filtrate was oxalic acid.

Attempted methylation of catenarin with methyl iodide. Catenarin (0·4 g.) was heated at 100° for 8 hours in a sealed tube with methyl iodide (1 g.) and sodium methoxide (1 g. sodium dissolved in 10 ml. methyl alcohol). After cooling the contents of the tube were acidified and the red precipitate collected; it consisted entirely of unchanged catenarin.

Action of hydrobromic acid on catenarin acetate. A mixture of catenarin acetate (0·5 g.) and a saturated solution of hydrobromic acid in acetic acid (15 ml.) was heated in a sealed tube at 100° during 8 hours. The contents of the tube, which were red in colour owing to hydrolysis, were poured into water, and after boiling for a few minutes to coagulate the precipitate the latter was collected and recrystallised from alcohol. The product had an indefinite M.P. 215–220° and was clearly a mixture. (Found: Br, 6·3 %. $C_{15}H_9O_5Br$ requires: Br, 22·9 %.) It was found impossible to isolate a homogeneous bromo-compound from this product by crystallisation. In a second experiment an unsuccessful attempt was made to purify the crude reaction product by acetylation and fractional crystallisation of the acetate; no definite separation was effected in six recrystallisations.

Efforts to improve the method by varying the conditions met with no success.

Absorption spectra.

The absorption of catenarin, hydroxycatenarin, helminthosporin[1] and cynodontin in the visible region of the spectrum is shown in Fig. 1 (p. 562). The curves refer to solutions in concentrated sulphuric acid, the concentration in each case being approximately $0{\cdot}5 \times 10^{-4} M$. The close similarity of the curves in their essential features, indicates the close relationship existing between these compounds and may be regarded as strong support for the formulae advanced for catenarin and hydroxycatenarin. (The absorption spectra of 1-methyl-3:5:8-trihydroxyanthraquinone and the related 1:4:6-trihydroxyanthraquinone described by Dimroth and Fick [1916] differ widely from any of those here described.)

Summary.

1. Hydroxyanthraquinones have been found to be produced by various species of *Helminthosporium*, in addition to those previously examined, when grown in a culture medium containing glucose as the sole source of carbon. The mycelium of *Helminthosporium catenarium* Drechsler contains hydroxy*iso*-helminthosporin as the chief constituent of hydroxyanthraquinone nature. This substance is more appropriately named *catenarin* and its constitution as a β-(hydroxymethyl)-1:5:8-trihydroxyanthraquinone is supported by its oxidation to a hydroxycatenarin closely resembling cynodontin (1:4:5:8-tetrahydroxy-2-methylanthraquinone).

2. *Helminthosporium velutinum* Link also produces catenarin but in this case the mycelium was found to contain appreciable amounts of ergosterol.

[1] Graves and Adams [1923] condensed 3:6-dimethoxyphthalic acid and *m*-cresol to a dimethoxyhydroxy-2-methylanthraquinone which was demethylated to a substance, M.P. 227°, described as 1:5:8-trihydroxy-3-methylanthraquinone. The compound was obtained in small quantity and was not analysed. However, it should have been identical with helminthosporin, and through the courtesy of Prof. Adams, to whom we are greatly indebted, we have been able to compare the substance with natural and synthetic helminthosporin with the result that the three specimens have been found to be identical. Owing to the confusion caused by the different system of numbering, we overlooked this important work of Graves and Adams in our first communication on helminthosporin.

3. *Helminthosporium avenae* Eidam was found to produce cynodontin and ergosterol in small relative amount.

4. *Helminthosporium tritici-vulgaris* Nisikado yielded catenarin, a little helminthosporin and a new pentahydroxy-2-methylanthraquinone termed *tritisporin*. The constitution of this substance is discussed and it is regarded as a 1:3:5:8-tetrahydroxy-6- (or 7-) (hydroxymethyl)anthraquinone.

We desire to express our sincere thanks to Mr W. K. Anslow for much technical assistance during the preparation of the crude pigments.

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LXXX. THE IRRADIATION OF A TURBOT VITAMIN A CONCENTRATE WITH HOMOGENEOUS ULTRA-VIOLET RADIATION.

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(*Received March 1st, 1934.*)

THE fact that concentrates of vitamin A may contain a chromogenic substance which under special conditions can be made to give rise to an increase in the Carr-Price units of the concentrate has been reported from time to time especially during the earlier work on the irradiation of vitamin A. Hawk [1929] and Norris and Church [1930] reported that exposure to air and light had this effect. Drummond [1930] however was unable to obtain similar results. Heilbron *et al.* [1931] found that in liver oils, which with the antimony trichloride test showed a preponderance of the 572 $m\mu$ chromogen over that of the 606 $m\mu$, an increase in strength of the 606 $m\mu$ occurred after several months' storage together with a small increase in strength of the extinction coefficient of the oils at 328 $m\mu$. In the author's work it has been found possible with a turbot concentrate containing 60 % vitamin A to increase both the blue units and the absorption coefficient at 328 $m\mu$ above the data recorded for the purest vitamin A by Carr and Jewell [1933].

EXPERIMENTAL.

A Hilger E_3 all-quartz spectrometer with sector photometer and a condensed copper spark to supply the light source were used to determine the absorption spectra of the solutions used. An air-cooled quartz mercury vapour lamp was used as source of ultra-violet radiation. The lamp was used throughout in the "end on" position and placed as close as possible to the vitamin A solution which was contained in a quartz test-tube and kept stirred during the experiments by a magnetic stirrer; irradiation was thus accomplished in the complete absence of air. A sheet of "uvirol" glass transmitting within the range 300–390 $m\mu$ was interposed between the solution and the lamp and all solutions were irradiated with this radiation. The value of E is calculated from the relation $\log I_0/I = Ecd$ where C is expressed in mg./ml., d in cm.; that of the blue values is calculated for Lovibond blue units for 1 mg./ml. as in the paper of Woolf and Moore [1932]. All solutions were irradiated and their absorption spectra determined in spectroscopic alcohol. After irradiation the solutions were evaporated at 50° *in vacuo* and concentrated for E and blue value readings, the blue values being determined in chloroform in the usual manner, the reading being taken immediately after addition of the antimony trichloride reagent.

A turbot concentrate prepared by Dr T. Moore of Cambridge was used in these experiments. It was found that with solutions containing 0·0011 % vitamin A an exposure of 3 hours caused only a small decrease in E and blue units and that solutions containing 0·00011 % vitamin A, in the case of this

particular concentrate, were readily affected by short exposures; the latter concentration was therefore used throughout this investigation.

After 1 minute's irradiation both *E* and blue units have decreased, and the absorption band of the blue solution of the non-irradiated concentrate at 565 $m\mu$ has changed to a band at 570 $m\mu$. After 3 minutes' irradiation *E* and blue units have further decreased and the blue solutions show a band at 575 $m\mu$. On further irradiation both *E* and the blue units increase until a maximum is reached after 21 minutes' irradiation, the absorption band of the blue solutions being 575 $m\mu$ and the absorption band in the ultra-violet at 328 $m\mu$ slightly flattened out. After 28 minutes' irradiation the blue units and *E* have fallen, selective absorption having almost disappeared, the band of the blue solutions with antimony trichloride now reading 570 $m\mu$. Further irradiation caused general absorption and very low blue values.

During irradiation a product has thus been formed which as regards *E* and blue units does not differ from vitamin A. The behaviour of the concentrate is certainly evidence of its lack of homogeneity and that the substance absorbing at 565 $m\mu$, which was evidently in excess in the concentrate, is transformed into one absorbing at 575 $m\mu$; further the change involves both *E* and blue units in definite proportions, so that if the substance absorbing at 575 $m\mu$ is without biological activity, it might readily simulate vitamin A. It is also noteworthy that the concentrate was so readily influenced by dilution as regards its reaction under ultra-violet radiation.

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LXXXI. NOTE ON THE ALLEGED REVERSAL OF THE DENATURATION OF SERUM-ALBUMIN.

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(*Received March 1st, 1934.*)

THE claim to have effected the reversal of the denaturation of serum-albumin [Anson and Mirsky, 1931] is so contrary to general experience that it is worthy of close examination. The method consists in dissolving the denatured protein in dilute acid and neutralising with dilute sodium hydroxide solution at room temperature. A portion of the albumin treated in this way crystallises like untreated albumin and behaves similarly in the precipitin reaction [Miller, 1933]. At first sight this evidence appears convincing but it is necessary to consider the various steps in the process and the assumptions involved very critically, since the conclusions reached are of some importance in the study of proteins.

EXPERIMENTAL METHODS.

Serum-albumin.

The serum-albumin used was prepared from horse plasma. Globulins were removed by precipitation with 18 % of anhydrous sodium sulphate at 34°, and crystallisation of the albumin was effected by cautious addition of acetic acid to the filtrate in the ordinary way. Crystallisation of albumin is usually carried out in ammonium sulphate filtrates, but the sodium sulphate process is equally effective. The crystalline albumin was filtered off on cloths, lightly pressed and dialysed in cellophane bags. The resulting solution was brought to p_H 7 and filtered through a sterile Seitz filter. The resulting 800 ml. of sterile 10 % solution of albumin was used as a stock preparation in these and other experiments.

Electrometric p_H *determinations.*

A compact micro-quinhydrone electrode assembly was devised (Fig. 1). The cell is readily made by drawing out a piece of 7·5 mm. bore glass tubing and bending the ends round into the form of a U-tube. The bottom horizontal portion of the tube is filled with 3 % agar saturated with KCl and acts as a salt bridge. In one limb is placed a buffer solution of known p_H and in the other the solution to be investigated. About 0·3 ml. of solution is sufficient for a determination. A little quinhydrone is added to each limb and platinum wire electrodes of the usual form are placed in each limb, being supported by corks resting on the rims of the limbs. A Cambridge portable potentiometer completes the simple apparatus.

Effect of p_H *on denaturation.*

A neutral 0·4 % solution of serum-albumin in 0·8 % saline was titrated with 0·1 *N* HCl, and 4·5 ml. samples of different p_H values were withdrawn at various stages of the titration. For the p_H determinations the quinhydrone vessel described above was used. The samples were placed in 5 × 0·5 in. tubes in a rack immersed in a tank of boiling water from which the burner had just

been removed. The tubes were so arranged in the rack that samples of similar p_H were not adjacent. When the tubes had been in the hot water for 3 minutes they were transferred to a bath of cold water. The precipitates, when present, were filtered off and protein determinations on the filtrates were conducted by means of the phenol reagent of Folin and Ciocalteu. In Fig. 2 are plotted the

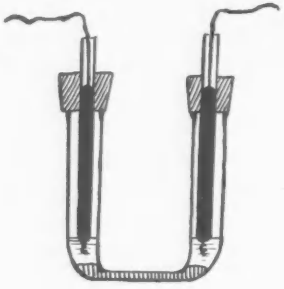

Fig. 1.

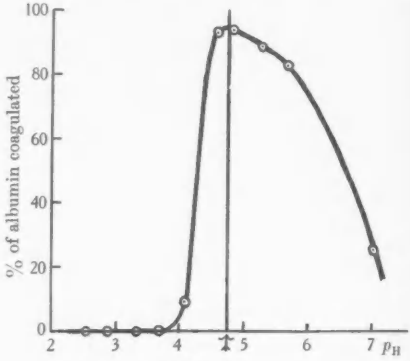

Fig. 2.

Fig. 1. Quinhydrone electrode vessel.

Fig. 2. Effect of p_H on heat-coagulation of albumin. (The vertical line, indicated by arrow, indicates the isoelectric point.)

amounts of precipitation at different levels of p_H. It will be observed that coagulation has been maximum in the region of the isoelectric point (p_H 4·5 to 5·0) and falls away slowly on the alkaline side but very steeply on the acid side. Thus, taking points equidistant from the isoelectric point (say p_H 4·75), at p_H 5·5 over 80 % of the protein is coagulated, whereas at p_H 4·0 only 9 % is precipitated. Heating at reactions more acid than p_H 4·0 does not effect coagulation when the solution is cooled and brought to the isoelectric point.

Effect of salts.

It is well known that coagulation of proteins on heating does not occur in the absence of salts and this is shown in the following experiment. The stock 10 % solution of albumin was diluted with distilled water giving a final concentration of 0·2 % albumin. Twelve samples were taken and their reactions adjusted with 0·02 *N* HCl to p_H values ranging from 4·1 to 7·2. The samples were heated in a boiling water-bath for 15 minutes but no coagulation occurred. A small amount of salt was added to each of the cooled tubes without there being any coagulation, but when the tubes were reheated coagulation occurred, being almost complete in the samples of p_H from 4·6 to 5·0. Electrodialysed albumin is said to coagulate without added salt [Adolf, 1926]. The effect of salts on protein coagulation was not taken into account by Anson and Mirsky.

Modifications of Anson and Mirsky's experiment.

Unless the experimental conditions, especially p_H, are carefully selected it is not possible to repeat Anson and Mirsky's experiment, and some care is necessary in finding the appropriate conditions, since in their paper no p_H

measurements are recorded. The following experiment is of some interest and will be described in detail.

A 2 % solution of serum-albumin in 0·8 % saline was rendered acid (p_H 2·3) by addition of hydrochloric acid and heated for 4 minutes in a boiling water-bath. The solution whilst still hot was divided into three portions. One portion was brought to the isoelectric point by addition of sodium hydroxide to the hot solution and then immediately cooled (Part I), a second portion was brought to the isoelectric point and allowed to remain hot for 2 minutes before cooling (Part II), whilst the third portion was allowed to remain hot for 2 more minutes, then cooled and finally brought to the isoelectric point when cold (Part III).

Part I, which had been brought to the isoelectric point and immediately cooled was in the form of a granular precipitate; Part II which had been heated for 2 minutes at the isoelectric point was in the form of a gummy coagulum; whilst Part III, which had been heated in acid solution and cooled before being brought to the isoelectric point, was a perfectly clear solution. The method of reversal of denaturation described by Anson and Mirsky was applied to Parts I and II only, since Part III was already totally in solution. Part I dissolved readily in dilute hydrochloric acid giving a clear solution but Part II gave a highly viscous gel with which efforts at reversal were quite unsuccessful. When the acid solution of Part I was again brought to the isoelectric point by addition of dilute sodium hydroxide at room temperature a turbidity was produced, and, on addition of an equal volume of saturated ammonium sulphate, there was a considerable amorphous precipitation. Now when an equal volume of saturated ammonium sulphate solution was added to Part III the protein remained soluble and on cautious addition of dilute acetic acid in the usual way the albumin crystallised out in fine rosettes of needles, no amorphous material being detected microscopically. If the granular precipitate (Part I) be centrifuged down and ground by an agate pestle in an agate mortar some 40 % dissolves up in the supernatant fluid.

Comment on these experiments is made in the following section, but a point raised by Miller [1933] must be referred to here. He mentions the fact that, since the solubility of native albumin in half-saturated ammonium sulphate solution near the isoelectric point is limited, the precipitate obtained is not necessarily all denatured protein. As a matter of observation however the addition of an equal volume of saturated ammonium sulphate to serum-albumin at the isoelectric point (p_H 4·8) results in a considerable change in p_H (to p_H 5·2) and at this reaction undenatured albumin is soluble.

Using the disulphide reaction [Walker, 1925] and observations of optical rotatory power [Hewitt, 1927] evidence of reversal of denaturation was not obtained.

Discussion.

At the outset we are faced by the significant fact that, in order to observe the reversal of denaturation, it is necessary to adhere rigidly, not only to the conditions essential for the reversal, but also to Anson and Mirsky's methods of preparing the denatured albumin. The exact repetition of their experiments is rendered difficult by their neglect to record the p_H values of their solutions.

The first two methods employed by Anson and Mirsky for preparing denatured albumin, namely precipitation with trichloroacetic acid and with acidified acetone, need not be considered for the moment as there is no satisfactory evidence that the albumin has been denatured. The third method consists in warming the albumin in acid solution and adjusting the reaction of

the hot solution to the isoelectric point. If immediately cooled the precipitate may be redissolved in dilute acid preparatory to the reversal of the denaturation. This is effected by the addition of 0·1 *N* NaOH to the solution at room temperature until the isoelectric point is reached. It may seem that the partial neutralisation with dilute caustic soda at room temperature is not a process likely to effect a reversal of denaturation. The dissolving of the precipitate in dilute acid would seem more likely and, indeed, Northrop [1931] has reactivated a small portion (up to 1 %) of inactivated pepsin by treatment with acid. Actually however the crux of the matter has appeared at an earlier stage. The fundamental difficulty lies in the exact definition of a denatured protein. Anson and Mirsky's [1931] definition, "All denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali," is not of general application, but it will serve our present purpose if accepted with suitable qualifications.

It is obvious that in order to prove the possibility of reversing the denaturation process it is essential to the argument to prove satisfactorily that the protein has in fact been denatured. Now Anson and Mirsky assume, without presenting any evidence to support the assumption, that when heated at 100° in acid solution for 4 minutes albumin is quantitatively denatured. It is a fact however that serum-albumin, after being treated in this way, then cooled down to room temperature and the reaction adjusted to the isoelectric point, gives no evidence of being denatured. The albumin is still soluble in water and dilute salt solutions at the isoelectric point, it is still soluble in 50 % ammonium sulphate and may be crystallised in the usual way. These facts would be explained by Anson and Mirsky as being due to the reversal of the denaturation during the neutralisation of the cold acid albumin solution. In order to avoid this reversal Anson and Mirsky neutralise the acid solution whilst it is still hot, and under these conditions protein is precipitated at the isoelectric point. They assume that reversal of denaturation is avoided by neutralising the hot instead of the cold solution. Reversal of the denaturation is now effected by dissolving the precipitate in dilute acid and then neutralising the cold solution. In this way some two-thirds of the original amount of protein may be recovered in the undenatured form. Anson and Mirsky's explanation however fails to explain why there should be quantitatively complete recovery of undenatured protein in the experiment mentioned above where the albumin heated in acid solution is cooled before neutralisation. Since the reversal process, namely the neutralisation of the cold acid solution, has been identical in each case the different yields of undenatured protein must be due to differences in the denaturation process. Anson and Mirsky suggest that the simplest explanation of their partial recovery of undenatured protein is that there are two effects of heating protein solutions, a reversible and an irreversible denaturation. There is however no necessity to introduce this complication. Let us consider for a moment a further modification of the experiment. If the hot albumin solution when brought to the isoelectric point, instead of being at once cooled, is kept hot for a minute or two longer, the character of the precipitate changes, and the denaturation cannot be reversed. (It should be emphasised that a longer period of heating the acid solution does not have this effect.) The simplest explanation of all the facts and observations is that the heating of the albumin in acid solution has little, if any, denaturing effect. Heating at the isoelectric point however results in very rapid denaturation, so that when the hot acid solution of albumin is brought to the isoelectric point, there is an immediate precipitation of granular floccules. These floccules consist of a shell or skin of denatured protein surrounding a core of undenatured albumin and when ground up in an agate mortar some 40 %

of the precipitate is dissolved. If the granular precipitate be immediately cooled, it dissolves readily in dilute acid, and when again neutralised the undenatured protein remains dissolved, whereas the denatured portion retains its characteristic properties, for example it is insoluble in half-saturated ammonium sulphate solution and is amorphous. If the experiment be varied by keeping the isoelectric solution hot for a minute or two before cooling, the denatured skin of the granular floccules can no longer protect the core and the protein in the interior of the floccules becomes denatured as well. This completion of denaturation is accompanied by a change in the appearance of the precipitate which now becomes more gummy. It is mentioned specifically by Anson and Mirsky that the portion of the protein (about one-third) which has not been reversed in the first instance, cannot be reversed by subsequent treatment. This portion corresponds to the denatured skin mentioned above and in the present experiments it was found that the longer the interval between the adjustment of the reaction to the isoelectric point and the cooling, the less protein could be recovered in the reversed condition. So far therefore there is no evidence that conflicts with the view that protein denaturation is an irreversible process and there is no evidence of two kinds of denaturation, reversible and irreversible.

At this point it is necessary to consider critically the evidence presented by Miller [1933] in support of the possibility of reversible denaturation. Using the precipitin reaction he concluded that native and "reversed" serum-albumin were similar but were different from denatured albumin. This putative evidence is however rendered void by the fact that the denatured protein he used was not a comparable control to the "reversed" albumin. The "reversed" albumin was prepared by Anson and Mirsky's method and the reversed portion had not therefore been denatured, whilst the "denatured" protein had been subjected to further heating processes at the isoelectric point, and was thus truly and irreversibly denatured.

It is clear therefore that the claim to have reversed the heat-denaturation of serum albumin has not been confirmed.

Summary.

1. No evidence for the possibility of reversing the denaturation of serum-albumin was found.

2. Serum-albumin may be heated in acid solution without being appreciably denatured.

3. In experiments purporting to demonstrate partial reversal of denaturation evidence is lacking that the albumin recovered had been denatured.

The author is indebted to Dr R. G. White for his encouraging interest.

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LXXXII. THE IDENTIFICATION OF AMINO-ACIDS BY MEANS OF 3:5-DINITROBENZOYL CHLORIDE.

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(*Received March 1st, 1934.*)

THE present investigation arose as a result of an attempt to prepare an easily obtainable crystalline derivative of oxidised glutathione. The author [1933] has already shown that oxidised glutathione fails to give a dinaphthalenesulphonyl derivative with naphthalene-2-sulphonic chloride, the latter being reduced to naphthalene-2-sulphinic acid.

An attempt was made to prepare the *N*-dibenzoyl derivative of oxidised glutathione by shaking it with benzoyl chloride and alkali according to the usual Schotten-Baumann method. On acidifying the product, a sticky mass was obtained together with a copious evolution of hydrogen sulphide.

It was obvious that the peptide was being decomposed by the alkali, and hence it seemed advisable to cut down the time of benzoylation to a minimum, a condition which might be accomplished by using 3 : 5-dinitrobenzoyl chloride. Both on the Lapworth theory of alternating polarities [Lapworth and Shoesmith, 1922] and on the more modern electronic theories this should behave as a highly reactive acid chloride. Unfortunately no product could be isolated in the case of GSSG, but this method of benzoylation using 3 : 5-dinitrobenzoyl chloride was extended to many other amino-acids with success.

It was found to be an exceedingly rapid and efficient method, pale yellow or colourless crystalline derivatives, having sharp melting-points, being usually obtained. The method consists in dissolving the amino-acid in excess of alkali and adding the calculated quantity of finely powdered 3 : 5-dinitrobenzoyl chloride. The mixture is shaken vigorously for about two minutes. The dinitrobenzoyl chloride dissolves almost instantaneously and on acidification the 3 : 5-dinitrobenzoyl derivative separates. In most cases practically no 3 : 5-dinitrobenzoic acid is formed provided that the acid chloride is not in excess.

All the monoamino-monocarboxylic acids examined gave crystalline *N*-3 : 5-dinitrobenzoyl derivatives, which on hydrolysis with hydrochloric acid gave 3 : 5-dinitrobenzoic acid and the original amino-acid.

The behaviour of the monoamino-dicarboxylic acids was rather unexpected. In the case of aspartic acid no product was isolable, but a considerable quantity of 3 : 5-dinitrobenzoic acid was produced. A formaldehyde titration of the filtrate showed that much of the aspartic acid remained unchanged. Glutamic acid also reacted only partially with 3 : 5-dinitrobenzoyl chloride, but in this case a certain amount of the 3 : 5-dinitrobenzoyl derivative was actually isolated. As previously stated no product could be isolated from GSSG.

It seems from this that the NH_2 group in the monoamino-monocarboxylic acids is highly reactive since it reacts rapidly and more or less completely with

3 : 5-dinitrobenzoyl chloride in the presence of sodium hydroxide. The mono-amino-dicarboxylic acids (and some other "acidic" amino-acids such as tyrosine) appear to have a much less reactive NH_2 group since much (or all) of the 3 : 5-dinitrobenzoyl chloride is hydrolysed before any condensation can be effected.

This observation is substantiated by the fact that whereas aspartic acid gives no product, and glutamic acid reacts only partially, asparagine and glutamine (which are less acidic) react completely with one[1] molecule of 3 : 5-dinitrobenzoyl chloride giving high yields of the corresponding mono-dinitrobenzoyl derivatives, practically no 3 : 5-dinitrobenzoic acid being formed. Furthermore *meso*diaminosuccinic acid gives a theoretical yield of the corresponding di-3 : 5-dinitrobenzoyl derivative.

The apparent lower reactivity of the NH_2 group in the "acidic" amino-acids compared with the reactive NH_2 group in the "neutral" amino-acids towards 3 : 5-dinitrobenzoyl chloride in alkaline solution may perhaps be due to the different state of ionisation of this group at the p_H employed. As the reaction is a very rapid one such differences might be detected, whereas in the slower ordinary benzoylations using benzoyl chloride, there is opportunity for an equilibrium to be disturbed.

Whatever the cause, this difference in behaviour has permitted of a smooth and complete separation of certain classes of amino-acids. For instance, glycine has been separated from a mixture of aspartic acid and glycine, the latter reacting preferentially with 3 : 5-dinitrobenzoyl chloride. The separation was not impaired by the presence of large quantities of sodium salts. It is advisable, but not necessary, to remove ammonium salts. They do not interfere with the reaction between 3 : 5-dinitrobenzoyl chloride and glycine, but some of the acid chloride may be wasted as 3 : 5-dinitrobenzamide. The latter, however, owing to its insolubility in sodium hydroxide does not contaminate the product.

It has not yet been definitely established whether there is any reaction between 3 : 5-dinitrobenzoyl chloride and gelatin in alkaline solution, but large quantities of the protein interfere with the reaction between the acid chloride and glycine. A weight of gelatin equal to the weight of glycine can however be present without any deleterious effect.

Glycine has also been separated from glutamic acid and asparagine from aspartic acid. Although glutamic acid does form a derivative, the glycine derivative is formed preferentially.

An efficient separation has also been effected between glycine and leucine, owing to the fact that 3 : 5-dinitrobenzoylleucine is precipitated from alkaline solution by dilute acetic acid, whereas 3 : 5-dinitrobenzoylglycine is not. Other possibilities in this direction become evident from details given in the experimental part.

Cystine reacted very rapidly with 3 : 5-dinitrobenzoyl chloride to give di-(3 : 5-dinitrobenzoyl)-cystine which was characterised by the formation of a gel in aqueous or alcoholic solutions.

One would expect a different type of derivative in the case of the thiolamino-acids. To determine the reaction of the SH group alone thiolacetic acid was used. A rapid reaction involving the SH group took place as shown by the disappearance of the nitroprusside reaction. On acidification an oil was obtained, which solidified on long standing and from its reactions appeared to be *S*-3 : 5-dinitrobenzoylthiolacetic acid. Wertheim [1929] was the first to use 3 : 5-dinitrobenzoyl chloride for the identification of simple mercaptans. His method however was

[1] There is no reaction between 3 : 5-dinitrobenzoyl chloride and the acid amide group as shown by absence of reaction with acetamide.

different from the above in that he used small quantities of pyridine as a catalyst instead of sodium hydroxide and found that it was necessary to fuse the reactants. It is obviously an advantage to use sodium hydroxide as the amino-acids are usually soluble in this medium. It should be pointed out that he did not use 3 : 5-dinitrobenzoyl chloride in conjunction with the amino group.

3 : 5-Dinitrobenzoyl chloride reacts with cysteine to produce what appears to be the *S*-derivative containing a free NH_2 group. Unlike the *N*-derivatives it does not possess a sharp melting-point. It is a light brown powder which is not easily recrystallised and cannot be recommended for identification purposes.

No reaction could be effected between tyrosine and 3 : 5-dinitrobenzoyl chloride. Neither phenol nor salicylic acid reacted with this reagent in alkaline solution.

Guanidine gives an almost theoretical yield of di-(3 : 5-dinitrobenzoyl)-guanidine. This derivative is insoluble in NaOH and so does not interfere with the identification of the amino-acids by this method.

The three aminobenzoic acids react almost instantaneously with 3 : 5-dinitrobenzoyl chloride, but homogeneous products are not obtained in the case of the *o*- and *p*-compounds. This problem will be discussed elsewhere.

The 3 : 5-dinitrobenzoyl derivatives cannot be analysed satisfactorily by the micro-Kjeldahl method, low results being obtained. Correct results were obtained by the micro-Dumas method.

Buehler *et al.* [1933] have recently described the 3 : 5-dinitrobenzoates of certain amines; these are salts and are distinct from the derivatives described above. The author was unable to obtain the 3 : 5-dinitrobenzoates of glycine and several other amino-acids, a result to be expected.

An attempt was made to prepare the 3 : 5-dinitrobenzoyl derivatives of the amino-acids using pyridine instead of NaOH as the condensing agent. On acidification in each case the same compound was obtained, which proved to be a very stable acid pyridine salt of 3 : 5-dinitrobenzoic acid ($2C_6H_3(NO_2)_2COOH.C_5H_5N$). This was confirmed by dissolving 3 : 5-dinitrobenzoic acid in excess of pyridine and acidifying, the same product being obtained. This compound had previously been prepared by Pfeiffer [1914].

3 : 5-Dinitrobenzoic acid and all the 3 : 5-dinitrobenzoyl derivatives here described gave an intense violet coloration with acetone and NaOH. This test could be used qualitatively to determine whether the 3 : 5-dinitrobenzoyl residue had entered the amino-acid molecule. The nature of this colour change is now being investigated.

Experimental.

3 : 5-Dinitrobenzoylglycine. 0·75 g. (1 mol.) of glycine was dissolved in 20 ml. *N* NaOH (2 mols.) and 2·32 g. (1 mol.) of finely powdered 3 : 5-dinitrobenzoyl chloride were added. The mixture was shaken vigorously in a stoppered bottle, whereupon the acid chloride dissolved almost immediately, the solution becoming deep red. The shaking was continued for about 2 minutes, the solution filtered if necessary and acidified with dilute hydrochloric acid. The dinitrobenzoyl derivative crystallised out immediately[1]. Yield 2·1 g. (80 % theoretical). It was recrystallised from water in long colourless silky needles: M.P. 179°. (Found: C, 37·6; H, 3·08; N, 14·66 %. $C_9H_7O_7N_3$, H_2O requires: C, 37·63; H, 3·13; N, 14·63 %. Dried at 100°. Found: H_2O 6·0 %. Calc. 6·27 %.)

[1] If an excess of 3 : 5-dinitrobenzoyl chloride were used, this appeared as 3 : 5-dinitrobenzoic acid in the precipitate produced on acidification and could be removed by shaking the precipitate in the cold with a mixture of five volumes of 50–60° light petroleum and two volumes of alcohol. The glycine derivative is insoluble in this mixture. If the acid chloride were not used in excess, it was usually found unnecessary to carry out this treatment.

Hydrolysis of 3 : 5-dinitrobenzoylglycine. The compound was boiled with HCl (2 vols. HCl, 1 vol. water) for 1¼ hours. On cooling 3 : 5-dinitrobenzoic acid crystallised out and was recrystallised from water: M.P. 207°. (Found: N, 13·1 %, $C_7H_4O_6N_2$ requires: N, 13·2 %.)

A formaldehyde titration of the filtrate showed that the hydrolysis to glycine was complete.

The 3 : 5-dinitrobenzoyl derivatives of the monoamino-monocarboxylic acids described below were prepared in a manner similar to the above. Each derivative dissolved in strong alkali to give a dark red colour discharged by acids. Dilute HCl was used for precipitation from the alkaline solution unless otherwise stated.

3 : 5-Dinitrobenzoyl-dl-*alanine.* Recrystallised from water in minute pale yellow needles: M.P. 177°. (Found: N, 14·88 %. $C_{10}H_9O_7N_3$ requires: N, 14·84 %.)

3 : 5-Dinitrobenzoyl-l-*leucine.* Precipitated by dilute acetic acid. Only slightly soluble in boiling water, from which however it can be recrystallised in long colourless needles. Recrystallised more readily from 50 % aqueous alcohol: M.P. 186–7°. (Found: C, 48·06; H, 4·6; N, 12·62 %. $C_{13}H_{15}O_7N_3$ requires: C, 48·0; H, 4·62; N, 12·92 %.)

3 : 5-Dinitrobenzoyl-d-*valine.* Precipitated by dilute acetic acid. Recrystallised from water in colourless feathery masses: M.P. 157–8°. (Found: N, 12·78 %. $C_{12}H_{13}O_7N_3$, H_2O requires: N, 12·76 %. Found: H_2O, 5·5 %. Calc. 5·47 %.)

3 : 5-Dinitrobenzoyl-l-*phenylalanine.* Precipitated by dilute acetic acid. In order to purify it, it was dissolved in alcohol, diluted with water until just cloudy and placed in the ice-chest to crystallise. Short colourless needles: M.P. 93°. (Found: N, 11·54; H_2O, 2·9 %. $C_{16}H_{13}O_7N_3$, 0·5 H_2O requires: N, 11·69; H_2O 2·45 %.)

3 : 5-Dinitrobenzoyl-l-*asparagine.* Asparagine reacts with one molecule of 3 : 5-dinitrobenzoyl-chloride to give the above compound using the method already described. Yield = 90 %. Recrystallised from water in very thin flat glistening leaflets: M.P. 196°. (Found: N, 17·19 %. $C_{11}H_{10}O_8N_4$ requires: N, 17·18 %.)

3 : 5-Dinitrobenzoyl-d-*glutamine.* Formed from one molecule of glutamine and one molecule of 3 : 5-dinitrobenzoyl chloride. Yield, 95 %. Recrystallised from water in colourless radiating needles: M.P. 217°. (Found: N, 16·38 %, $C_{12}H_{12}O_8N_4$ requires: N, 16·47 %.)

N-*3 : 5-Dinitrobenzoyl*-S-*benzylcysteine.* 1·05 g. of *S*-benzylcysteine were dissolved in 10 ml. of *N* NaOH and 1·15 g. of 3 : 5-dinitrobenzoyl chloride added. The mixture was shaken until all the acid chloride had dissolved. A strong mercaptanic odour was noticeable, owing presumably to the partial hydrolysis of the *S*-benzylcysteine by the alkali. The solution was filtered and acidified with dilute acetic acid, a white feathery crystalline precipitate being formed. Recrystallised from aqueous alcohol in long slender needles: M.P. 92°. It was subsequently found advantageous to carry out the reaction at 0°, in which case no mercaptanic odour was perceptible. The reaction was complete in a few minutes at this temperature. Yield, 90 %. (Found: N, 9·69; S, 7·26 %. $C_{17}H_{15}O_7N_3S$, $2H_2O$ requires: N, 9·52; S, 7·26 %.)

N-*3 : 5-Dinitrobenzoyl*-S-*ethylcysteine.* As for *S*-benzylcysteine. Precipitated by acetic acid. Recrystallised from aqueous alcohol in colourless needles: M.P. 65°. Hygroscopic. (Found: N, 12·26; S, 9·03 %. $C_{12}H_{13}O_7N_3S$ requires: N, 12·24; S, 9·33 %.)

3 : 5-Dinitrobenzoyl-l-*methionine.* Unlike *S*-ethyl- and *S*-benzyl-cysteine, methionine could be acylated at room temperature without any decomposition. Recrystallised from aqueous alcohol in slender silky needles: M.P. 94–5°; anhydrous substance has M.P. 150°. Very soluble in cold alcohol. (Found: C, 40·76; H, 4·08; N, 12·14; S, 8·98 %. $C_{12}H_{13}O_7N_3S$, 0·5 H_2O requires: C, 40·91; H, 3·98; N, 11·93; S, 9·09 %.)

3 : 5-Dinitrobenzoyl-l-*tryptophan.* 1 mol. of tryptophan reacted with 1 mol. of 3 : 5-dinitrobenzoyl chloride. An orange precipitate was produced on the addition of dilute acetic acid to the alkaline solution. Recrystallised from alcohol as an orange crystalline powder. Insoluble in boiling water. M.P. 233° (decomp.). (Found: C, 54·3; H, 3·54 %. $C_{18}H_{14}O_7N_4$ requires: C, 54·27; H, 3·52 %.)

Di-(3 : 5-dinitrobenzoyl) meso*diaminosuccinic acid.* 1 mol. of *meso*diaminosuccinic acid reacted with 2 mols. of dinitrobenzoyl chloride in the presence of 4 mols. of *N* NaOH. On the addition of dilute acetic acid to the alkaline solution a crystalline precipitate slowly formed. The filtrate gave no precipitate with HCl showing that no 3 : 5-dinitrobenzoic acid had been produced. The precipitate was insoluble in boiling water, alcohol, acetone, benzene and glacial acetic acid. It dissolved in alkali with the production of a deep red colour. In order to purify it, it was dissolved in NaOH,

water was added until the red colour was just discharged and dilute acetic acid was added to reprecipitate it as a colourless crystalline powder. Melted with decomp. 254°. (Found: N, 16·03 %. $C_{18}H_{12}O_{14}N_6$ requires: N, 15·67 %.)

Di-(3 : 5-dinitrobenzoyl)-d-*arginine*. 1 mol. of arginine hydrochloride reacted with 2 mols. of the acid chloride. Precipitated from the alkaline solution by dilute acetic acid. The product so obtained was boiled with alcohol and filtered from a small quantity of tarry residue. On the addition of water a colourless crystalline powder gradually separated. M.P. indefinite, shrinks and softens about 150°. (Found: C, 41·9; H, 3·69; N, 18·95; H_2O, 1·4 %. $C_{26}H_{19}O_{12}N_8$, 0·5 H_2O requires: C, 42·03; H, 3·51; N, 19·61; H_2O 1·58 %.)

Di-(3 : 5-dinitrobenzoyl)-l-*cystine*. 0·48 g. of cystine (1 mol.) dissolved in 8 ml. *N* NaOH reacted with 0·93 g. of the acid chloride (2 mols.). On acidifying the alkaline solution with dilute HCl a gummy mass was precipitated which became friable on standing. This was filtered off and purified by dissolving in hot alcohol, filtering and adding water until just turbid. The liquid was then warmed until just clear and allowed to stand. The whole mass set to a gel which was broken up, filtered and dried in a desiccator, giving a powder which gradually turned brown in the course of a few days. Darkened at 154°, melted with decomposition about 180°. (Found: N, 12·41, 12·54; S, 9·38, 9·67 %. $C_{20}H_{16}O_{14}N_6S_2$, $2H_2O$ requires: N, 12·66, S, 9·64 %. Loss of H_2O at 100°, 4·4 %. Cal. 5·1 %. Found for anhydrous substance N, 13·13 %. Calc. 13·37 %.)

The dry powdered product again formed a gel when dissolved in alcohol and diluted with water. A gel was also formed from aqueous acetone, dilute HCl and dilute acetic acid. Insoluble in benzene. The nitroprusside test was given after reduction with Zn and HCl.

Di-(3 : 5-dinitrobenzoyl)guanidine. 0·54 g. of guanidine sulphate was dissolved in 10 ml. *N* NaOH, 1·15 g. of powdered 3 : 5-dinitrobenzoyl chloride added and the mixture shaken for about 2 minutes. The guanidine derivative (insoluble in NaOH) was filtered off and recrystallised from alcohol in radiating clusters of colourless needles. Insoluble in water, moderately soluble in acetone, slightly soluble in benzene: M.P. 280° with blackening (shrinks about 215°). The filtrate from the reaction gave no precipitate with HCl, showing that all the acid chloride had reacted. (Found: N, 22·05 %. $C_{15}H_9O_{10}N_7$ requires: N, 21·93 %.) The substance was hydrolysed by boiling with 1 : 1 HCl for 1 hour. On cooling 3 : 5-dinitrobenzoic acid crystallised out.

Reaction between d-*glutamic acid and 3 : 5-dinitrobenzoyl chloride*. 0·73 g. (1 mol.) of glutamic acid was dissolved in 15 ml. of *N* NaOH. 1·15 g. (1 mol.) of powdered 3 : 5-dinitrobenzoyl chloride were added and the mixture shaken for 2 minutes. Nothing was precipitated on the addition of dilute acetic acid. Acidification with HCl gave an oil which crystallised on standing. After some time the crystalline precipitate was filtered off and the filtrate made up to 100 ml. It still contained unchanged glutamic acid. Using Sørensen's formaldehyde titration, 10 ml. of this solution required 2·1 ml. of *N*/10 NaOH. This indicated that 42 % of the glutamic acid was unattacked.

The precipitate was dissolved in cold alcohol (in which it was very soluble). Water was added until just cloudy, and the mixture was allowed to stand, whereupon 0·17 g. 3 : 5-dinitrobenzoic acid separated. The alcohol was evaporated from the filtrate and 3 : 5-dinitrobenzoylglutamic acid crystallised out. It was recrystallised from water as colourless needles. The substance softens and shrinks at 98–99°. (Found: C, 41·24; H, 3·26; N, 11·89 %. $C_{12}H_{11}O_9N_3$, 0·5 H_2O requires: C, 41·14; H, 3·28; N, 12·0 %. Loss in weight when dried at 100°, 2·4 %. Calc. 2·6 %. Found for anhydrous substance: N, 12·45 %. Calc. 12·32 %.)

It was subsequently found that a better separation of 3 : 5-dinitrobenzoic acid from the glutamic acid derivative could be obtained as follows. The reaction was carried out as above except that 1·5 g. of sodium acetate were added before the addition of the dinitrobenzoyl chloride. On acidifying with HCl, 3 : 5-dinitrobenzoic acid separated in crystalline form (0·53 g.). The filtrate on standing for a few minutes deposited 3 : 5-dinitrobenzoylglutamic acid (0·54 g.). This was then recrystallised from hot water.

Reaction between aspartic acid and 3 : 5-dinitrobenzoyl chloride. 0·66 g. of aspartic acid was dissolved in 15 ml. of *N* NaOH and 1·15 g. of powdered 3 : 5-dinitrobenzoyl chloride added and the mixture shaken for 2 minutes. No precipitate was produced on acidification with dilute acetic acid, but 0·5 g. 3 : 5-dinitrobenzoic acid separated at once on addition of dilute HCl. Formaldehyde titration showed that 50 % of the aspartic acid remained unchanged.

Separation of glycine from aspartic acid. A mixture of 0·38 g. of glycine and 0·66 g. of aspartic

acid was dissolved in 20 ml. of *N* NaOH. 1·3 g. of 3 : 5-dinitrobenzoyl chloride were added (an excess for the glycine present). The mixture was shaken, filtered and acidified with dilute HCl. The precipitate, which consisted of a mixture of the glycine derivative and 3:5-dinitrobenzoic acid, was dried and shaken with light petroleum-alcohol (5 : 2) mixture in the cold. The residue was recrystallised from water: M.P. 178°. The presence of large quantities of sodium acetate and other sodium salts did not impair the separation.

In the following mixtures the first named component was separated by a method similar to the above: asparagine from aspartic acid; glycine from gelatin (an equal weight); glycine from glutamic acid.

In presence of gelatin an excess of alkali was required. Also the product on acidification was usually oily, but crystallised if allowed to stand for some time. For the mixture of glycine and glutamic acid it is an advantage to have as little excess of 3 : 5-dinitrobenzoyl chloride as possible (calculated for the glycine).

Separation of leucine and glycine. A mixture of 0·33 g. of leucine and 0·19 g. of glycine was dissolved in 10 ml. of *N* NaOH. 1·15 g. of 3 : 5-dinitrobenzoyl chloride were added and the mixture shaken. On acidification with acetic acid 3 : 5-dinitrobenzoylleucine crystallised out. After standing for some time (to ensure complete precipitation) this was filtered off and recrystallised from aqueous alcohol: M.P. 186°.

Dilute HCl was added to the filtrate to precipitate the glycine derivative. Recrystallised from water: M.P. 178–179°.

Reaction between thiolacetic acid and 3 : 5-dinitrobenzoyl chloride. 0·92 g. of thiolacetic acid was dissolved in 20 ml. of *N* NaOH and shaken with 2·30 g. of 3 : 5-dinitrobenzoyl chloride. The nitroprusside test became negative almost immediately. On acidification either with dilute acetic or hydrochloric acid an oil was produced which solidified on long standing. The substance proved difficult to recrystallise. (Found for unrecrystallised substance: N, 10·3; S, 12·0 %. Calc. for $C_9H_{16}O_7N_2S$: N, 9·8; S, 11·19 %.) Melts with decomp. about 135°. Very soluble in alcohol and acetone, slightly soluble in benzene. When recrystallisation was attempted by dissolving in alcohol and adding water, an interchange of radicals evidently took place as the substance which crystallised out contained no sulphur and proved to be ethyl 3 : 5-dinitrobenzoate.

The product of the condensation was undoubtedly *S*-3 : 5-dinitrobenzoylthiolacetic acid. It was completely hydrolysed by boiling with 5*N* HCl for 30 minutes. On cooling 3 : 5-dinitrobenzoic acid separated out, and the filtrate gave a strong nitroprusside reaction.

Reaction between cysteine and 3 : 5-dinitrobenzoyl chloride. 0·79 g. of cysteine hydrochloride (1 mol.) was dissolved in 15 ml. of *N* NaOH and shaken with 1·15 g. (1 mol.) of the acid chloride. The nitroprusside test became negative almost immediately. The solution was filtered and a brown powder precipitated by the addition of dilute acetic acid. The product was purified by dissolving in alkali and reprecipitating with acetic acid. Melted with decomp. about 160°. Very soluble in alcohol and acetone, slightly soluble in water, insoluble in benzene. Hygroscopic. (Found: N, 13·31; S, 9·96 %. $C_{10}H_9O_7N_3S$ requires: N, 13·33; S, 10·1 %.)

On carrying out the reaction with 2 mols. of the acid chloride the same substance appeared to be produced. The reaction was also carried out in an atmosphere of nitrogen with identical results.

Summary.

1. 3 : 5-Dinitrobenzoyl chloride is recommended as a reagent for the rapid identification of many amino-acids, crystalline derivatives usually being obtained in good yield. The condensation is almost instantaneous in alkaline solution.

2. The acidic amino-acids appear to be less reactive towards this reagent. Under the conditions cited in the experimental part aspartic acid does not give a derivative. In this way glycine and several other amino-acids have been separated from aspartic acid.

Although glutamic acid does give a derivative, 3 : 5-dinitrobenzoylglycine is formed preferentially and a separation has been based on this fact.

No condensation could be effected with tyrosine in alkaline solution.

3. The 3 : 5-dinitrobenzoyl derivatives of certain amino-acids are precipitated from alkaline solution by dilute acetic acid. Others are not precipitated by this reagent, but only on the addition of dilute HCl. In this way glycine and leucine have been separated.

4. Guanidine gives a good yield of crystalline di-(3 : 5-dinitrobenzoyl) guanidine, which is insoluble in alkali.

5. Thiolacetic gives *S*-3 : 5-dinitrobenzoylthiolacetic acid by the above method. Cysteine also gives an *S*-derivative which is not, however, suitable for identification purposes.

I wish to thank Sir F. G. Hopkins for the kind interest that he has shown in this work.

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LXXXIII. THE RELATIONSHIP OF IRON WITH THE AGEING OF CELLS.

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The life of the multicellular organism is divided into certain phases by developmental events which occur during those periods. During embryonic and early post-embryonic existence cell division and cell growth are exceptionally prominent. As cell differentiation increases, cell growth and cell division progressively diminish and finally shrink to a minimum. After the cell has reached its adult stage, it ages and finally dies. Thus growth, differentiation, ageing and death are stages in a continuous process. As these are stages in a process the phases gradually blend into one another.

The results of the following investigation show, for the first time, that a distinct alteration in the chemistry of the cell occurs during development. An examination of the iron content of epithelial organs showed that the cells of an organ in an old animal contain appreciably higher quantities of iron than do those of a young animal. Estimations of copper under similar circumstances did not show such variations.

The iron content of the liver, kidney, testis—and in some cases of the lung and muscle tissue—of rats, guinea-pigs, rabbits, dogs and cats was estimated, all tissue utilised in this investigation being freed from blood before chemical examination by the following methods. Immediately after death a cannula was inserted through the left ventricle into the aorta, and Ringer's solution at body temperature was infused under considerable pressure until the venous fluid was entirely free of blood. Perfusion was carried out in this manner for 20 to 30 minutes at least, and in order completely to exsanguinate the liver and kidney this was followed by further perfusion through the portal vein in the case of the liver. and through the abdominal aorta in the case of the kidney, after all the vessels except the renal artery had been ligatured. The completeness of exsanguination was controlled by histological examination.

The iron determinations were made according to Warburg's [1927] method by which iron may be detected in quantities as small as 5×10^{-4} mg. 50 mg. of thoroughly disintegrated tissue were placed in a platinum crucible and incinerated by heating for 10 to 12 hours at 800° in an electric furnace. 1 ml. of 0·1 *N* HCl was poured into the crucible containing the ash, and 0·1 to 0·2 ml. of the resultant solution was examined for iron. In all, this investigation, which was made at the Charité, Berlin, includes the examination of tissues from 125 animals. The greater number of estimations were on rats; but the study of other animals was sufficient to show that in them the results were similar to those in the rat.

The iron content of pulmonary tissue showed greater variations than that of the liver, kidney and testis. These irregularities may possibly be explained by the inhalation of iron during life. Hence the examination of lung tissue was carried out only in a relatively small number of rats. Similarly the spleen may contain an

appreciable amount of iron as a result of the destruction of erythrocytes, and therefore this organ was excluded.

Is the increase in the iron content of aged cells of special significance or is it dependent on the destruction of blood during life? The latter possibility can be excluded for the following reasons.

1. Histological examination did not show this increased iron to be due to haemosiderosis, and further the increased iron occurred regularly in organs, such as in the testis, in which haemosiderosis does not usually take place.

It might be claimed however that the histochemical methods for the demonstration of iron are too crude to demonstrate the minute amounts present in the cells, or to show variations when these are present.

2. The lower values for iron found in the tissues of young animals and the high values of the old are definite and constant. This is not only true in different animals of the same species but also in animals of various species during corresponding phases of development.

3. If the observed increase in the iron content of the tissues of old animals were due to iron deposited in tissues following blood destruction, then this increase should be continuous throughout life; such is not the case. According to our results the increase in the iron content of the cells in old adults is clearly associated with a definite developmental phase. The increase occurs within a short time and remains at the new level throughout the remainder of the animal's life. This increase of the cell-iron must therefore be of some functional significance.

A study of the iron values of the organs of the rat is illuminating. In the rat, the iron content of the kidney and testis is the same throughout the first 10 months of life (Fig. 1) and in the majority of these animals, even throughout the whole of the first year of life. In a certain proportion of animals, the iron content begins to increase between 12 and 15 months after birth but more frequently after 15 months.

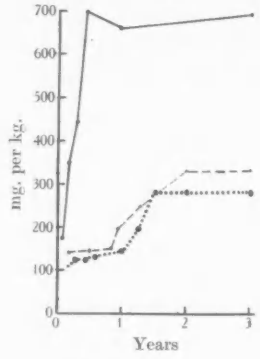

Fig. 1. Rat.

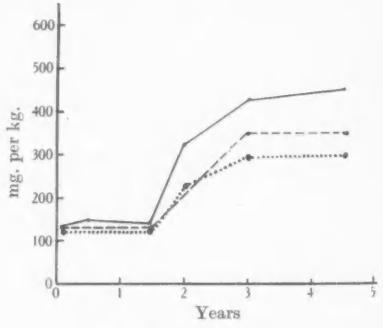

Fig. 2. Guinea-pig.

—— Liver. – – – Kidney. • • • Testis.

The values reach their maximum at $1\frac{1}{2}$ years, while only in a small proportion of cases is this higher level delayed until the second year of the animal's life. Throughout the remainder of the rat's existence (in all 3 to 4 years) the iron values remain the same. The increase of iron in the liver occurs at about the third month, and maximum values are obtained by the end of the fifth month.

Thus the change from the low level prevalent in the tissues of the young to the high values obtained in the old animals occurs in 3 to 6 months, a relatively short time. Indeed, this rapid increase occurring as it does at a definite interval indicates that this phenomenon is due to some physiological process and is not the result of such a coincidence as the deposition of haemosiderin in the organs.

A further point is also of importance. The rise in iron values occurred in all the types of animals examined and at the same period, that is the middle period of the animal's life.

Since the duration of life of guinea-pigs and rabbits is longer than that of rats, the increase of tissue-iron does not begin before the end of the second year (guinea-pig) or even later (rabbit) (Fig. 2 and Table I). In the liver the increase

Table I. *Rabbit.*

Iron contents of organs at various periods of life in mg. per kg. dried substance—average values.

	Days				Months		Years			
Age	1	3	28	33	$2\frac{1}{2}$	3	1	$1\frac{1}{2}$	2	6
Liver	1400	—	—	180	160	—	240	200	200	700
Kidney	500	500	—	150	160	—	160	170	—	350
Testis	—	—	125	—	—	—	120	—	125	260

generally begins somewhat earlier than in the other epithelial organs, but the difference is not as great as in the rat. In dogs and cats of 4 to 5 years of age the low values found in the young are still present, and a definite increase in liver-iron does not occur until the seventh year. In the young animals, the results are closely grouped together (from 100 to 180 mg. per kg. of dried tissues, Tables II and III). As already noted, this is true in animals of various species (Table IV). The maximum values of the old animals show somewhat greater variation, but for the different species the readings fall into a definite group (200 to 500 mg.). In the liver the maximum values may be higher—up to 800 mg. (Tables II, III, IV B).

Table II. *Rats.*

Iron content of kidney of rats (mg. per kg. dried substance).

No. of animal	Under $\frac{1}{4}$ year (young animals)	No. of animal	Over $1\frac{1}{2}$ years (old animals)
1	170	1	268
2	182	2	200
3	162	3	215
4	90	4	435
5	120	5	476
6	107	6	500
7	115	7	326
8	184	8	300
9	170	9	300
10	148		
11	180		
12	123		
13	115		
14	120		
15	140		
Average	142		335

Table III. *Rat.*

Iron content of testis of rats (mg. per kg. dried substance).

No. of animal	Under $\frac{3}{4}$ year (young animals)	No. of animal	Over $1\frac{1}{2}$ years (old animals)
1	96	1	205
2	115	2	355
3	103	3	330
4	85	4	218
5	135	5	294
6	142	6	272
7	157	7	250
8	135	8	264
9	110	9	526
10	128	10	280
11	180	11	182
12	145	12	260
		13	210
		14	400
		15	335
		16	276
		17	270
		18	410
		19	180
		20	345
		21	200
		22	210
		23	270
Average	130		283

Table IV.

Average iron content of organs of animals (mg. per kg. dried substance).

	Animal	Liver	Kidney	Testis
A. Young animals				
	Rat	180	140	130
	Rabbit	170	155	125
	Guinea-pig	145	140	125
	Cat	200	125	—
	Dog	200	150	115
B. Old animals				
	Rat	About 700	335	280
	Rabbit	Up to 700	400	260
	Guinea-pig	450	350	280
	Cat	Up to 800	—	—
	Dog	Up to 600	—	—

As to the iron content of the organs of young animals, Bunge [1898] found that certain animals, *e.g.* rabbits, are born with a relatively large store of iron. But according to the results of this investigation the tissue-iron of rabbits rapidly decreases in amount during the first weeks of post-embryonic life to the same low level which young animals of other species already have from birth (Table I).

Reports have already been published of the estimation of iron in the organs of animals. Very often differences have been found to exist between different species. For instance, the amounts obtained in mice and rats were much larger than those in guinea-pigs and rabbits. The probable explanation is that for small animals, such as mice and rats, the older ones have been utilised in order to obtain sufficient material, while in the case of larger animals, such as guinea-pigs and rabbits, even the organs of the very young are sufficient for analysis.

The iron values are independent of the food intake of the animals. This has been proved by depriving rats of vitamin B-containing food. In spite of this and the consequent considerable loss of weight, the iron content was always found to correspond with that of other animals at the same period of life.

The iron values in the organs of the young are so constant and the increase during age so regular that it is possible to determine the age group of an animal by iron estimations, preferably of the kidney or testis. Low iron values in the testis of rats—below 160 mg. per g. of dried tissue—definitely indicate that the animal is under $1\frac{1}{2}$ years of age, while quantities above 180 mg. suggest that the animal's age is over 10 months and most likely over 15 months. The intermediate period between 10 and 15 months cannot be determined with certainty because it constitutes the transition from the period of low iron values to that of the high values.

The iron content of the striped muscle of young animals is about the same as that found in epithelial organs, especially in the kidney and testis. From this fact we have to conclude that the iron is present not only in cells but also in the paraplasmatic substances in which muscle tissue is very rich. The rise of the iron content in old animals only occurs in the epithelial organs, not in the striped muscle. Therefore the increase of the iron content is probably due to a new iron compound.

Summary.

1. The iron content of the epithelial organs in the animals investigated in this series (rats, guinea-pigs, rabbits, cats and dogs) is constant.

2. A similar constant iron value is obtained in the various organs of the same animal, in the organs of animals of the same species, and as between the organs of animals of the various species. Only in the liver are the variations appreciable, and even here these variations are relatively small.

3. At a definite period of life and within a short time, the iron content of epithelial organs is increased up to 200 %. The same iron value is then maintained until death.

4. This newly acquired iron is probably a compound other than those already present in the cell and must be associated with the ageing of the cell. Hence the longer the life period of the animal the later does the increase occur. The middle period of life can be considered the period during which the increase in all iron occurs.

5. The iron content of the organs of young animals is so constant and its increase at a definite period is so regular that from the iron content of the organs of an animal, especially the kidney and testis, it is possible to determine the age group of the animal from which the tissue has been taken. The cells of epithelial organs having a high iron content can be considered as in the decline of life.

6. Since the increase in the iron content of organs is so regular a feature, the phenomenon must have some biological significance.

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LXXXIV. THE BIOLOGICAL VALUES OF PROTEINS.

V. THE COMPARATIVE BIOLOGICAL VALUES OF THE PROTEINS OF WHOLE WHEAT, WHOLE MAIZE AND MAIZE GLUTEN, MEASURED BY THE GROWTH OF YOUNG RATS.

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In the previous paper of this series [Boas-Fixsen and Jackson, 1932] experiments were described in which the biological values of the proteins contained in various wheat and maize products were determined by the "balance sheet" method, using adult rats. These experiments revealed no difference in value, for maintenance of nitrogenous equilibrium, between the proteins of whole wheat and whole maize. It was pointed out that this result had an important bearing on the theory attributing pellagra, which in its epidemic form is closely associated with the consumption of maize, to an amino-acid deficiency.

A survey of previous work on the subject suggested that, whereas the proteins of wheat and maize might be of equal value for maintaining nitrogenous equilibrium during a short period, a disparity might be revealed by tests in which the growth of young rats during long periods was the criterion employed. The present paper describes experiments of this nature.

The growth of young rats has been used extensively for determining the relative nutritive values of proteins, and the method has been extended to include longevity and reproduction as criteria, by McCollum *et al.* [1921]. Osborne and Mendel [1916; 1920] and Osborne, Mendel and Ferry [1919] have made a detailed study of proteins using this method and devoted much attention to the precautions necessary in this type of experiment in order to permit of trustworthy deductions. They emphasised the importance for growth of the total food intake apart from the protein intake. They considered that ideal conditions for comparison of the biological values of different proteins were attained when rats of similar weight grew at equal rates and ate about the same amount of similarly constituted diets, the only variable in the diets being the nature and proportion, and hence the amount, of protein ingested. This amount was held to be in inverse proportion to the biological value [Osborne and Mendel, 1916].

Subsequently Osborne and Mendel adopted the value of the ratio $\frac{\text{g. gain in weight}}{\text{g. protein ingested}}$ as a numerical expression of the biological value for any given protein. The value of this ratio was found to increase at first as the proportion

of protein in the diet was raised, reaching a maximum after which it declined. This maximum value, obtained under conditions which favoured the most economical use of the protein, was taken as the most trustworthy estimate of its biological value [Osborne, Mendel and Ferry, 1919]. This method involved a long series of experiments with diets containing different proportions of each protein investigated and, besides being very laborious, did not conform to the ideal conditions set out above, for the total consumption of food was not the same in parallel experiments.

Most investigators who have used this method have calculated the weight increase per g. protein ingested from the results of experiments in which the different proteins were present at the same level in the diets and not necessarily at the level at which the above ratio has the maximum value [see also Osborne and Mendel, 1920].

Mitchell [1924, 1; 1928] has offered an explanation for the fact that, for any given protein, the value of the ratio $\frac{\text{g. gain in weight}}{\text{g. protein ingested}}$ (subsequently referred to in this paper as Ratio X_1) has been found to increase with increasing protein intake. He has suggested that in this expression no account is taken of the protein requirement for maintenance, apart from the requirement for growth, and that the former is a greater proportion of the total intake when this is small; in order to obtain uniformity of intake in comparative experiments, he devised his paired feeding method [Mitchell and Beadles, 1930].

Results reported later in this paper tend to support Mitchell's view and show further that if allowance is made for the protein required for maintenance, the above expression tends to give a constant figure for the biological value for growth of any given protein, irrespective of the intake.

Technique.

The experiments here described were confined to diets containing wheat or maize products as source of protein. Most observations were made with whole wheat or whole yellow maize, a few only being made with white maize for comparison with the yellow. The protein content of the maize was low (from 9 to 11 % on the dry weight) compared with that of the wheat, which was relatively high (16·4 %). It was not therefore possible to construct a diet, within the capacity of the animals' appetite, containing sufficient protein to support normal growth derived from whole maize. To see whether, in any case, normal growth could be obtained on maize proteins, diets were prepared containing the gluten of yellow and white maize at various levels up to 23 %. These glutens are by-products obtained in the manufacture of corn starch and consist of a mixture of maize-glutelin and zein, the water-soluble globulins and a proportion of the glutelin having been washed away [Osborne and Mendel, 1914]. They do not therefore represent true concentrates of maize-proteins.

The provision of the vitamin B complex has always been a difficulty in experiments of this type. In work on cereals reliance has been placed either on the cereals themselves for the source of supply (in which case the results are complicated by their differing contents of these vitamins) or on whole yeast. The drawback of a yeast supplement is the addition by this means of small amounts of extraneous protein of good quality [Osborne and Mendel, 1919; Mitchell, 1924, 3].

Osborne and Mendel used chiefly "protein-free milk", while Hoagland and Snider [1926; 1927] used yeast extracts, all of which supplements contain an appreciable amount of nitrogen. In the present work a dilute acetic acid extract from brewer's yeast was used from which the proteins had been precipitated by boiling [Chick and Roscoe, 1929].

The diets were designed to support a subnormal degree of growth, which should be limited only by the amount of protein present. In this connection it is noteworthy that Osborne and Mendel [1916], in a comparison of caseinogen, lactalbumin and edestin, found that the differences

between the nutritive values of these proteins for growth could not be detected if the amounts present corresponded to more than 12·5 % of the total calories. It is found, however, that with diets which contain a very low percentage of protein, the food intake and growth are poor, and the results are very irregular. A 10 % level of protein (on the dry weight) was therefore chosen for the diets with whole wheat and whole yellow maize; the white maize, however, was so poor in protein that it was not possible to make a diet containing more than 9 % protein.

The few recorded estimations of the biological values of proteins of maize and wheat using the criterion of Osborne and Mendel are summarised in Table I. The values are not consistent, those for whole wheat-proteins vary from 1·1 to 2·1; a value of 1·2 was found for whole maize-protein.

Table I. *Nutritional value of proteins of wheat and maize for supporting growth in young rats.*

Source of protein	% protein in diet	Duration of exp. days	Initial wt. of rats g.	Source of vitamin B complex	No. of rats	Gain in body wt. per g. protein ingested	Observer
Whole wheat	10	30	43	Yeast concentrate	6	2·1	Hoagland and Snider [1927]
	10	60	43		6	1·6	
,,	10	70	60–70	Wheat	3	1·3	Osborne and Mendel [1920]
,,	8	70	,,	,,	6	1·1	
,,	5	70	,,	,,	4	1·1	,,
,,	10	56	35–60	Dried yeast	10	1·7	Morgan [1931]
Whole maize	9	56	35–60	,,	4	1·2	,,
	9	63	—	Maize (in 3 exps. vit. B extract)	8	1·2	Maynard *et al.* [1923]

Materials used.

Table II gives the description and analysis of the cereal products used as sources of protein. The nitrogen was estimated by the Kjeldahl method and the crude fibre and fat by the methods of Weender and Dormeyer, respectively [see Lunge and Keane, 1911]. The carbohydrate was calculated by difference, and the calorie value of the diet as made up was estimated from these data. The composition of the diets, their water and protein contents and their calorific values are set out in Table III. The diets were fed chiefly in the raw condition, but some observations were made using the whole wheat and whole maize diets after mixing with 40–60 % of water and steaming for one hour.

Table II.

		Composition (% of air-dry weight)							Calories
Material	Description	Water	Total protein (N ×6·25)	Zein*	Ash	Fibre	Fat	Carbohydrate (diff.)	(kg.-cal.) per g. (dry wt.)
1. Whole wheat	Soft, red English	14·3	14·1	—	1·4	1·6	1·9	66·7	3·40
2. Whole yellow maize†	S. American	(1) 14·0 (2) 15·2	(1) 9·3 (2) 9·8	3·3 (34)‡	1·2	1·4	2·8	69·6	3·43
3. Whole white maize	S. African	10·7	8·4	2·6 (32)‡	1·1	1·9	4·1	73·8	3·66
4. Yellow maize gluten	Prepared from S. American yellow maize	8·7	27·0	11·7 (43)‡	0·6	1·3	5·2	57·2	3·92
5. White maize gluten	Prepared from (3)	6·9	41·5	22·0 (53)‡	0·7	0·7	5·6	44·6	3·95

* (N soluble in 90 % alcohol) ×6·25. † Two samples used. ‡ % of total protein.

Table III. *Composition of diets used.*

Cereal	Wheat	Yellow maize	White maize	Yellow maize-gluten				White maize-gluten	
% protein approx.	10	10	9	9	13	17·6	22	14·5	23
Wheat	690	—	—	—	—	—	—	—	—
Yellow maize	—	1058	—	—	—	—	—	—	—
White maize	—	—	1053	—	—	—	—	—	—
Yellow maize-gluten	—	—	—	312	469	624	815	—	—
White maize-gluten	—	—	—	—	—	—	—	333	535
Salts	25	25	25	25	25	25	25	25	25
Butter fat	35	35	35	35	35	35	35	35	35
Cottonseed oil	23	—	—	20	13	6	—	20	12
Corn starch	363	—	—	709	557	409	225	688	483
% water in diets	12·9	(1) 13·3* (2) 14·0*	10·4	12·3	11·1	10·9	10·1	11·9	10·1
% protein in dry diet ($N \times 6{\cdot}25$)	9·73	(1) 9·77* (2) 10·1*	9·04	9·02	13·0	17·6	22·5	14·5	(1) 23·0 (2) 24·5†
% total cals. from protein	8·90	(1) 9·58* (2) 9·89*	8·65	7·66	11·3	15·5	20·2	12·3	(1) 20·2 (2) 21·5†
Calorie value per g. dry diet	4·37	4·08	4·18	4·71	4·61	4·54	4·46	4·70	4·56

* A sample of maize containing lower percentages of water and protein was used in earlier experiments.
† Diet used for last 10 days of experiments.

Digestibility. The digestibility of the protein in the whole maize and whole wheat diets was determined as follows. Young rats were kept in pairs in metabolism cages and fed on the experimental diet for a preliminary period of 3 days; the faeces were collected during a subsequent period of 4 days and the daily faecal output of nitrogen (N) and the nitrogen intake (I) estimated. The daily faecal output of endogenous nitrogen (E) was also determined for each pair of rats during a period in which a nitrogen-free diet was fed [see Boas-Fixsen, 1930]. The undigested nitrogen of the protein tested is then equal to N – E, and the digestibility to $\left(1 - \frac{N - E}{I}\right) \times 100$. The average figures, calculated from four experiments on each diet, were as follows: wheat, raw 84 %, cooked 85 %; maize, raw 92 %, cooked 89 %. Cooking did not therefore appear to alter the digestibility of these proteins, although it increased the food intake (see below, p. 597). The slightly higher values obtained for maize are probably not significant.

Mitchell [1924, 2] also found the proteins of whole maize and whole wheat to be equally digestible, but the value determined, 94 %, was higher.

There is some disagreement among different authorities as to the allowance that should be made for nitrogen of endogenous origin in the faecal nitrogen [see Schneider, 1934]. According to Mitchell [1924, 2], E is proportional to the amount of food taken, and in the expression N – E, the value of E should be adjusted to correspond with the actual food intake in the experiments with the proteins under consideration, which is almost invariably greater than in the trials on nitrogen-free diets. If such an allowance be made in the present case, the digestibility values are slightly raised, and those for the wheat diets slightly more than the corresponding figures for the maize diets, since rather more was eaten of the wheat than of the maize diet. The figures become: wheat-protein, raw 88 %, cooked 91 %; maize-protein, raw 94 %, cooked 92 %.

Experimental.

Black and white rats from the Lister Institute stock were used. At first, litters of young rats were placed on experiment at 21–22 days of age, irrespective of the body weight, which varied from 34 to 54 g. Subsequently it was found more satisfactory to keep the young rats on the ordinary breeding diet until they weighed 50 g. or over. The experiment proper lasted for 10 weeks; the first week was treated as a preliminary period to enable the animals to become accustomed to the diet and calculations were made only on the results of the subsequent 9 weeks. Each rat was kept in a separate cage of the type used in

vitamin B work, a fine wire grid being placed over the coarse wire mesh of the cage floor to prevent loss of food. The legs of the cage rested on a sheet of blotting-paper which covered a tray containing peat. This facilitated the recovery of scattered diet and enabled trustworthy figures to be obtained for the intake of food.

The diets were mixed and stored as air-dry powders. The daily ration was weighed accurately to 0·5 g., added to the residue of the preceding day and mixed with water to a paste. At the end of the experiment, or at the end of any of the periods into which it might be desired to subdivide it, the residue was collected in a weighing-bottle dried at 100° and weighed to 0·1 g.

The diets to be compared were distributed as evenly as possible among littermates. Each rat received, five times a week, a supplement of 4–6 drops (85 mg.) of cod-liver oil to provide vitamins A and D, and 1·5 ml. of yeast fraction "5" as source of the B-vitamins (see above, p. 593); 1 ml. of this extract was derived from 0·5 g. of the yeast, and contained 0·0065 g. nitrogen. The total nitrogen obtained from this source during the 9 weeks period of the observation amounted to 0·46 g.

Results.

The performances of the different groups of rats and a description of their general condition are given in Table IV. In no case was the growth normal, although this standard was approached with diets containing 22–24 % maize-gluten, where the average gain in weight was 100–109 g. in 9 weeks.

Table IV.

	Material	% protein in diet (dry wt.)	No. of rats used	Condition of rats at end of 10 weeks on diet	Increase in wt. g. Variation	Increase in wt. g. Mean	Total food intake (dry) g. Variation	Total food intake (dry) g. Mean	Total caloric intake (mean) kg.-cal.	Protein intake g. (mean)	Ratio X_1 = g. gain in wt./g. protein intake
1.	Wheat	9·7	14	Good. No long silky hairs in fur	44–66	55	324–510	424	1820	40·5	1·36
2.	Wheat (cooked)	9·7	9	Excellent	42–94	72	364–624	489	2140	47·6	1·51
3.	Yellow maize	9·9	16	Not so good as (1). Coat poorer	32–79	52	327–567	406	1660	40·4	1·29
4.	Yellow maize (cooked)	9·9	9	Good. Coats nearly normal	26–87	65	285–631	471	1920	47·2	1·38
5.	White maize	9·0	6	As group 3	19–85	59	323–628	501	2095	45·3	1·30
6.	Yellow maize-gluten	9·0	4	Poor	(−4)–(+17)	6	176–286	227	1070	20·4	—
7.	,,	13·0	5	Fair	6–78	24	213–285	261	1200	34·0	0·71
8.	,,	17·6	9	Good (Coats nearly normal)	31–74	58	287–505	413	1870	72·6	0·80
9.	,,	22·5	4	Good (Coats nearly normal)	83–128	100	475–666	550	2450	124·0	0·81
10.	White maize-gluten	14·5	7	Stools rather loose	52–77	64	387–520	460	2160	67·0	0·96
11.	,,	23·0*	3	,,	93–125	109	565–674	637	2900	148·0	0·74

* 24·5 for last 10 days.

The amounts of the food intake, protein intake and increase in body weight of rats receiving the whole raw wheat diet (10 % protein, Group 1) are of the same order as those of the animals on the similar diet made with whole raw yellow maize (Group 3). There is a similar correspondence in the results obtained with the cooked wheat and cooked maize diets (Groups 2 and 4). In these experiments, therefore, the values obtained for the biological value of the protein, as estimated by the ratio X_1, are comparable. The value for the protein

of whole raw wheat, 1·32, is slightly higher than for raw yellow maize, 1·29. In the six observations with raw white maize (Group 5, 9 % protein) the average food intake and growth were higher than for Groups 1 and 3; the growth per g. protein was the same (1·30) as for yellow maize.

Of the cooked maize and wheat diets (Groups 4 and 2) more was eaten and there was a larger increase in body weight, also the values of the ratio X were greater than for the raw diets, and that obtained for cooked wheat-proteins (1·51) was definitely larger than for cooked maize-protein (1·38). Since it is unlikely that the nutritive value of the proteins of these cereals is improved by steam cooking, it would appear that the palatability of the diets, apart from the quality of the protein, affected the values for biological value obtained by this method of calculation.

In the experiments with the maize-glutens, a much lower food intake and less growth occurred when the diets contained from 9 to 13 % protein. Only when 14–18 % protein was present did the food intake and weight increase reach the levels (413–460 g. and 58–64 g. respectively) obtained with 10 % protein derived from the whole grains. The amount of protein ingested in the experiments with the maize-gluten was much greater, and the values obtained for the ratio X_1 are correspondingly lower and vary from 0·71 to 0·96 (see Table IV).

Relation between protein intake and weight increase.

In Fig. 1, increase in weight is plotted against protein intake for each rat in the groups receiving diets containing 10 % protein from whole wheat and

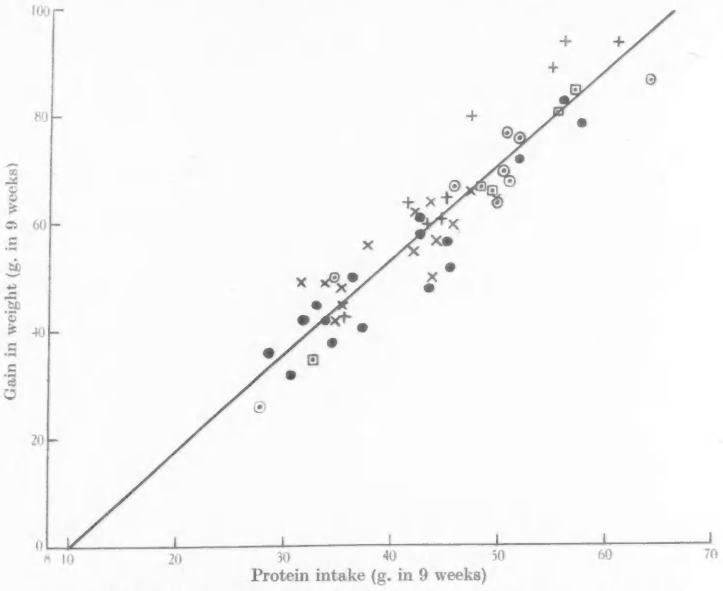

Fig. 1. Relation between gain in weight and protein intake for young rats on diets containing 9–10 % protein from whole wheat and whole maize.

× Wheat, raw. + Wheat. cooked. ● Yellow maize, raw
⊙ Yellow maize, cooked. ⊡ White maize, raw.

whole yellow maize, and 9 % protein from white maize. The experimental points lie grouped about a straight line, which, if produced backwards, cuts the base line at a distance from the origin corresponding to about 10 g. protein. Although it may seem unlikely that 10 g. protein could represent the maintenance requirements for rats whose average body weights varied from 60 to 100 g., yet if this amount is subtracted from the total protein ingested and the excess regarded as being used for growth requirements, the increase in weight is found to be proportional to this excess. The value of the ratio X_2 thus calculated $\left(\frac{\text{g. increase in weight}}{\text{g. protein ingested} - 10}\right)$ was found to be roughly constant in value, irrespective of food or protein intake.

10 g. protein in 9 weeks, together with the nitrogen (0·46 g.) derived from the B-vitamin supplement, provided a daily ration of (25·4 + 7·3) 33 mg. nitrogen, an amount which is about what might be expected for maintenance requirements. Mitchell and Carman [1924] found that about 30 mg. was the daily total nitrogen output on a "N-free" diet of young rats from 60 to 130 g. weight. Using their figure for the biological value of wheat-proteins (av. 67) the daily requirement of wheat-nitrogen to balance this expenditure would be about 44 mg. From the results of experiments with another series of rats, from 45 to 80 g. weight [Mitchell, 1924, 3] the endogenous daily nitrogen requirements can be calculated at from 17 to 23 mg. or about 30 mg. wheat-nitrogen.

Table V. *Biological values of the proteins of whole wheat and whole maize.*

Diet	No. of rats	Caloric intakes	Gain in weight / Prot. intake $=(X_1)$ Variations	Mean	Gain in weight / Prot. intake -10 $=(X_2)$ Variations	Mean	X_2 mean value
10 % wheat*	7	1415–1694	1·20–1·53	1·36	1·69–2·27	1·91	1·85 ±0·0256
	8	1837–2000	1·14–1·53	1·37	1·48–2·02	1·80	
	8	2026–2727	1·31–1·69	1·50	1·64–2·05	1·87	
10 % yellow maize*	10	1153–1505	0·93–1·45	1·22	1·46–2·03	1·76	1·73 ±0·0229
	6	1713–1846	1·10–1·47	1·31	1·61–1·87	1·69	
	9	2005–2574	1·29–1·52	1·40	1·61–1·90	1·74	
9 % white maize	5	1520–2626	1·07–1·50	1·30	1·44–1·81	—	1·65

* The figures in this Table are derived from all experiments made with these diets, both raw and cooked.

In Table V, which includes the results of all experiments with wheat and maize diets, both cooked and uncooked, values are given of the ratio X_2, obtained after subtraction of this "maintenance protein" from the denominator. Values are also given of ratio X_1, in which this correction is omitted. The data from which these ratios are calculated show great individual variation as regards food intake and gains in weight and the results from the 23 and 25 rats which received respectively the wheat and yellow maize diets are ranged in three groups according to the caloric intake. As this is increased, so the value of X_1 is found to increase, whereas that of X_2 remains approximately constant.

The values for X_2 calculated from experiments with the cooked wheat and maize diets are grouped together with the values derived from the experiments with the raw diets, because they showed no significant difference. The figures were: for wheat-proteins, raw 1·83, cooked 1·90; for maize-proteins, raw 1·72, cooked 1·74. As shown in Table IV, both intake, growth and the values for X_1 were greater for the cooked than for the raw proteins, but since the correction in the denominator, employed in calculating X_2, is proportionally less for the cooked than for the raw diets, the values for X_2 tend to become more nearly

identical than those for X_1 (wheat-proteins, raw 1·36, cooked 1·51; maize-proteins, raw 1·29, cooked 1·38).

The average value of X_2 obtained for whole wheat-protein, 1·85, is slightly higher than that for the whole maize-protein, 1·73. The difference between these mean values (0·12) is more than three times the probable error of this difference (0·0343); there is therefore a more than 20 : 1 chance that the difference found between the nutritive values of wheat- and maize-proteins for growth is significant. On the other hand, in comparison with the maize diet, the wheat diet contained a slightly lower proportion of protein relative to the total calories; and the protein may therefore have been utilised with slightly greater efficiency for that reason.

Maize-gluten. The results in Table IV indicate an inferiority of the maize-gluten diets as compared with those composed of the whole grain. For example, the rats in Group 8, receiving diets containing 17·6 % protein from yellow maize-gluten ingested food of about the same total calorie value (1870 kg.-cal.) and showed about the same average increase in weight (58 g.) as the rats in Group 3, receiving diets with only 10 % protein derived from the whole maize grain (1660 kg.-cal.; increase in weight 52 g.) and as those in Group 1 receiving a similar diet prepared from whole wheat (1820 kg.-cal.; increase in weight 55 g.). In the first case this amount of growth was supported by a total of 73 g. protein whereas in the second and third instances only 40 g. protein were needed.

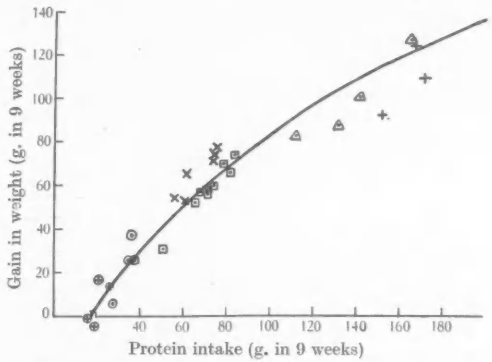

Fig. 2. Relation between gain in weight and protein intake for young rats on diets containing 9 to 23 % protein from maize-gluten.

		% protein
⊕	Yellow maize-gluten	9·0
⊙	,, ,,	13·5
⊡	,, ,,	17·5
△	,, ,,	22·0
×	White maize-gluten	15·0
+	,, ,,	23·0

Fig. 2 shows graphically the results of the whole series of experiments with the yellow and white maize-glutens. In this case the points appear to lie on a flat curve and no simple relation is shown between the weight increase and protein intake. It should be remembered, however, that in the experiments with whole wheat and whole maize illustrated in Fig. 1, the protein was fed throughout at approximately the same level (10 %) whereas, in the experiments

with the glutens, the protein level varied from 9 to 24 %. The form of the curve indicates that the gluten is utilised with less efficiency when present in the diets at higher levels.

The point at which the curve cuts the abscissa corresponds with an intake of about 18 g. protein in 9 weeks, which would suggest that the amount of maize-gluten-protein required for maintenance is greater than the amount required of whole maize-protein.

Since, however, this point for maize-gluten is determined mainly by experiments with diets containing over 14 % protein, this value for maintenance (18 g.) cannot fairly be compared with that obtained for the whole cereal diets (10 g.). The results of the experiments with 9 % protein from yellow maize-gluten appear, at first sight, to indicate that about 15–25 g. maintenance protein are required at this level also. It must, however, be remembered that, in these experiments (Group 6, Table IV), the caloric intake was very low, so that some protein may have been used to provide energy and the protein maintenance requirements raised on that account.

In the samples of white and yellow maize the proteins contained 32 % of zein (calculated from the amount of nitrogen soluble in strong (90 %) alcohol) and presumably about 68 % of glutelins and globulins [see also Osborne and Mendel, 1914]. The similarity in nutritive value between the total proteins of whole wheat and of whole maize indicates that the glutelin and globulins contained in the maize are able to supplement the deficiencies of the zein.

In the specimens of maize-gluten used, the proportion of alcohol-soluble protein was much greater than in the whole grain, and amounted to 43 and 53 % of the total protein in the yellow and white maize-glutens, respectively. Presumably the 57 to 48 % present of other maize-proteins retained in the gluten was insufficient to supplement this larger proportion of zein.

Summary.

1. An attempt has been made to measure the relative nutritive values of the proteins of wheat and maize for the growth of young rats, using the criterion adopted by Osborne and Mendel, *viz.* the gain in weight per g. protein ingested.

2. The proteins of whole wheat and of whole yellow and white maize administered in diets containing 9–10 % of these proteins were found to have nutritive values of about the same order, being 1·36, 1·29 and 1·30 respectively, as determined by the above method. After cooking, these cereal diets were taken better by the animals, and the nutritive value of their proteins appeared to be slightly greater (1·51 and 1·38 for wheat and yellow maize-proteins respectively).

3. Graphic representation of the above results indicated that, if an amount of protein (10 g.) were subtracted from the total protein ingested over the experimental period of 9 weeks, the ratio, growth/g. protein ingested, was roughly constant in value and independent of the food intake. This 10 g. might be supposed to represent "maintenance requirements" apart from those of growth. The value of the ratio $\frac{\text{g. weight increase}}{\text{g. protein ingested} - 10}$ would then indicate the biological value of the proteins for the growth of young rats.

4. The value of this ratio was 1·85 for the proteins of wheat, and 1·73 for those of maize, when present in the diet at a 9–10 % level. There was no difference between values obtained for the cooked and raw cereals.

5. The nutritive value of "maize-gluten" was found to be inferior to that of the proteins of the whole grain, doubtless due to the greater proportion of zein contained.

6. The results of the above experiments, showing only a slight difference between the nutritive values of the proteins of whole wheat and whole maize for support of growth in the young rat, is in accord with previous work showing their similar values for maintenance of the adult rat.

We wish to express our gratitude to Dr H. Chick for her constant help and criticism during the investigation. Two of us (M. A. B-F. and J. C. D. H.) are indebted to the Medical Research Council for grants and to the Lister Institute for hospitality. We wish also to acknowledge the help given by Messrs Brown and Polson and Messrs MacKean of Paisley in providing us with the maize products, and by Mr Walter Acton of the latter firm for much information concerning them. We also acknowledge the assistance of Messrs J. and H. Robinson in providing the samples of whole wheat used.

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LXXXV. BRADYCARDIA IN THE VITAMIN B_1-DEFICIENT RAT AND ITS USE IN VITAMIN B_1 DETERMINATIONS.

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In an earlier paper [Drury *et al.*, 1930] it was shown that rats suffering from vitamin B_1 deficiency had low heart rates. When vitamin B_1 was administered rapid cures resulted, the effect on the heart rate being proportional to the amount of vitamin allowed. It was suggested that this action on the heart might form the basis of a very convenient, rapid and accurate method for estimating the vitamin. Vitamin B_2 and various other food constituents were without similar action. During the past three and a half years this method has been in continuous use in this laboratory where it forms our usual routine method of test in vitamin B_1 assays. As a result of this further experience—during which several hundred tests in all have been carried out—we have had the opportunity of collecting further evidence as to the advantage of the method and have examined its degree of accuracy when carefully checked against other standard methods of vitamin B_1 assay. In the present paper we present a typical set of results, illustrating the use of the method in practice. The principal examples which we give relate to tests on the materials selected for investigation in 1930 by the sub-committee appointed in this country to examine methods and standards for vitamin B_1, preparatory to the adoption of the League of Nations International Standard. These materials were (1) the "activated acid clay" standard, and certain specimens of (2) yeast, (3) marmite and (4) wheat germ. Parallel determinations on each of these four materials were carried out by us by three other methods, *viz.* (1) cure of convulsions in rats, (2) growth rate in rats, and (3) cure of "polyneuritis" (head retractions) in pigeons, and are here compared with our results by the bradycardia method. These data were included in the memorandum on antineuritic vitamin B_1, placed before the International Conference on Vitamin Units and Standards of the Permanent Standards Commission of the Health Organisation of the League of Nations, London 1931.

PART I.

Determination of vitamin B_1 by the bradycardia method.

1. *Technique.*

The electrocardiographic records needed in this method are taken by a procedure already outlined [Drury *et al.*, 1930]. The unanaesthetised rat is held down firmly on its back on a board, its head being held by an adjustable metal clamp and its paws and hind-legs fixed by slip nooses attached to cleats at the

side of the board. The electrodes consist of two small needles, one being placed under the skin of the right fore-leg the other at the lower end of the thorax. These needles are connected to a Matthews's portable electrocardiograph on which permanent photographic records are then taken.

The method of carrying out an assay is as follows. Young rats weighing about 40 g. are placed on a basal diet of

Sugar	60
Arachis oil	15
"Light white casein"	20
Salt mixture	5
Autoclaved marmite (1 hr., 15 lbs., p_H 10) ...	6
Cod-liver oil	1 drop per rat per day.

After about 3 weeks, when the animals are beginning to decline in weight, electrocardiograms are taken. By this time the heart rate should have fallen from

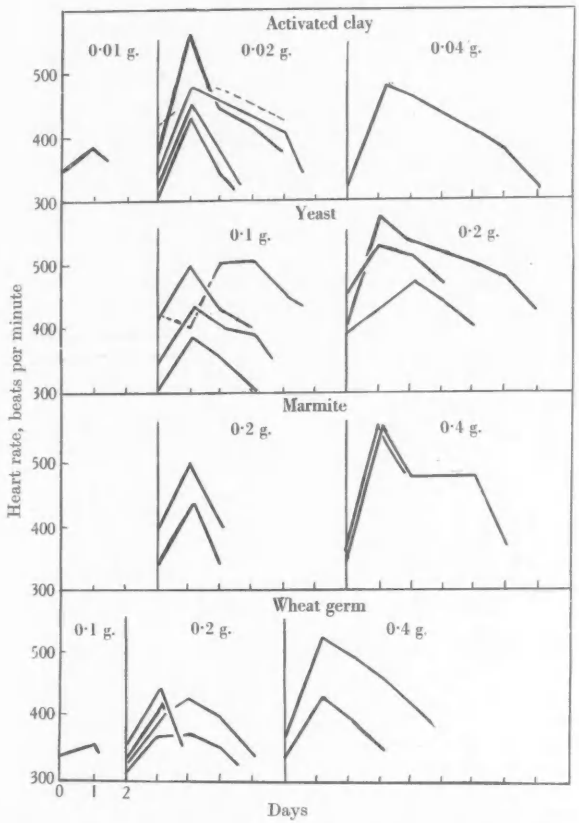

Fig. 1. Heart rate after administration of different sources of vitamin B_1.

Table I. *Duration of cures.*

Material	Dose fed g.	Individual duration of cure (days)	Average duration of cure (days)	Relative activity calculated from curve
Activated clay	0·01	1·5	1·5	
	0·02	2·5, 4·5, 2·5, 4, 4	3·5	100
	0·04	6	6	
Yeast	0·1	3, 3, 3·5, 4·5	3·5	21
	0·2	4, 5, 6	5	16·5
				18·8
Marmite	0·2	2, 2	2	(6)
	0·4	5, 5	5	8·3
				8·3
Wheat germ	0·1	1	1	5·5
	0·2	2, 2, 4, 3·5	3	9
	0·4	5, 3	4	6·5
				7

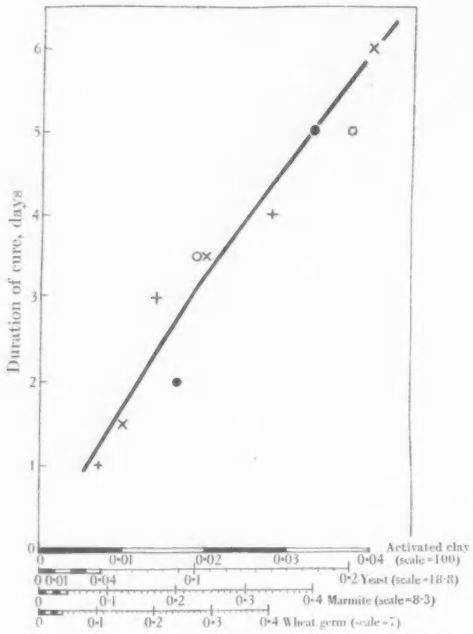

Fig. 2. Bradycardia method: dose-effect curve.

× = Activated acid clay standard.
o = Yeast.
• = Marmite.
+ = Wheat germ.

To determine the scales for the unknown, the points for the standard are first plotted; unknowns are next plotted roughly at approximately the correct scale; and then the value for each point of the unknown is calculated against the standard curve. The average of these values (shown in Table I) is used in re-plotting for final comparison, as above.

the normal rate of 500–550 per minute to a rate of about 350 per minute. As soon as the rate has fallen to this figure a single dose of the substance to be tested is fed. The animal is replaced in its cage and given basal diet and water *ad lib.* as before. After 24 hours its electrocardiogram is again taken. If the rhythm has not increased a larger dose must be tried. With a sufficiently large dose the rate is suitably increased and is measured every 24 hours, or better twice daily, until it has fallen again to about the same low value as when the dose was administered. The increase in rate and the time over which the increase lasts are roughly proportional to the dose given. Several levels of unknown are tested in this way, with about 4 to 6 animals in each group, and comparison is made simultaneously with the amount of standard reference material needed to produce the same effect. A dose-effect curve may be plotted from which relative activities may be determined for intermediate readings, whenever the "effects" are not quite identical (*cf.* Fig. 2). Cures of about 4 days' duration are found to be the most suitable basis for comparison. A quick method, when approximate results suffice, is to find the amount of supplement needed to give the smallest detectable effect on the heart rate lasting about 1–2 days. If doses lasting longer than about 4–5 days are given errors may be caused by apparently incomplete utilisation of the vitamin. This is especially the case with sources of moderate activity and less so with active concentrates.

2. *Illustrative results.*

In the tests taken for description here the "activated clay" standard was compared with yeast, marmite and wheat germ fed simultaneously at two or three different levels of activity. Fig. 1 gives the daily records of the heart rates after dosing. It is seen that 0·02 g. of activated clay is roughly equivalent to 0·1 g. of the yeast, or slightly more potent than 0·2 g. of marmite or of wheat germ, and other doses in proportion. The average durations of cure at each level of dosage are worked out in Table I, and the relative activities of the four substances can then be calculated more accurately from the combined dose-effect curve, Fig. 2. The activities so determined, taking activated clay as 100, are: yeast 19, marmite 8, wheat germ 7. These results will be found to agree excellently (Table VII) with those determined by the three other standard methods. We now turn to a discussion of the latter, giving various recommendations as to the technique which we have found most advantageous, and shall return later to a comparative discussion of the bradycardia method.

PART II.

Cure of convulsions in rats.

1. *Method.*

Our technique is a modification of that described by Smith [1930].

Diet. The basal diet consists of

"Light white casein"	18
Salt mixture	4
Arachis oil	10
Sucrose	58
Autoclaved brewer's yeast	10
Dried brewer's yeast	0·4
Cod-liver oil	1 drop per rat per day.

The small addition of brewer's yeast is made because in our experience a larger percentage of the rats develop convulsions consistently when a trace of vitamin B_1 is provided in the diet, which is in conformity with the conclusions of Sherman and Sandels [1931] and Sebrell and Elvove [1931]. The 0·4 % level was chosen because after numerous trials it was found to give the best results. Sugar is used in place of starch in order to avoid risk of refection and also because we found that the substitution tended to increase the percentage incidence of convulsions. On this diet over 50 % of animals can be relied upon to develop convulsions at the regular time.

Preparation of animals. Young rats, either albino or piebald, weighing about 70–80 g. are placed on the above diet. After about 6–8 weeks, appetite having fallen considerably, "polyneuritic" symptoms begin to appear. The rat develops leg weakness and drags his limbs, the gait is "wobbly" and there is evidence of inco-ordination (swollen red paws said to be indicative of deficiency of Reader's "vitamin B_4" have not been seen). As a criterion for the presence or absence of definite "polyneuritis" the rat is picked up and suspended by the tail, and rotated between the thumb and finger for a second or two: if convulsions follow the rat is ready for use.

Method of test. A single dose of the substance to be tested is administered to each of a series of such rats. If, as in the work under description, the substance is a foodstuff, it is fed *per os*. If it is an active concentrate it is probably better to inject it into the peritoneum. Provided a sufficient dose has been given the animal is cured within 24 hours. If it is not cured in 2 days the test is regarded as negative, and a larger dose is then given. Animals which die within 24 hours of dosing are neglected, as when they are in the proper condition they should live for at least two days with the "polyneuritic" symptoms. If the dose has been sufficient and the animal is cured, the number of days' respite from convulsions is noted.

Normally, using the minimum dose of standard needed to effect a proper cure, the cure lasts about 5–6 days. With certain substances, however, such as marmite it tends to last slightly longer, *e.g.* about 8–12 days: this may be due to the slower rate of absorption.

Calculation of results. The agreement between duplicate animals as regards the duration of cure is very close. For example a 6-day dose will produce cures lasting somewhere between 4 and 9 days in the great majority of animals in any given group. Occasionally, however, an animal may be encountered which behaves abnormally, appearing to remain cured or partially cured over a protracted period: such animals are neglected in computing the results of the assay. In a few cases these same animals have also failed to respond normally during the depletion period, and coprophagy was suspected—it cannot always be obviated even with screened floors.

If an approximate idea only is desired of the potency of different preparations under test, it can be obtained with great ease and using very few rats by simply comparing the minimum doses of each needed to produce a definite cure—such cures usually lasting about 5 days. This method is especially useful in determining roughly the activity of solutions during a process of concentration.

At about this minimum level, however, results tend to be more variable and partial cures are more frequent. More precise results are obtained therefore by providing sufficient of the supplement to give cures lasting from about 6 to 12 days and feeding several dosage levels of both unknown and standard, using several duplicate rats at each level. Doses of standard and unknown are then compared which give approximately the same duration of response. To allow for slight

differences a dose-effect curve (Fig. 3) is plotted, but it is always advisable to restrict comparisons to doses of unknown and standard which give similar magnitudes of cure. At medium levels the duration of cure is found to be very nearly proportional to the weight of vitamin given, so that the method of calculating activity from "day-dose" (dose given/average duration of cure) is reasonably accurate. As higher levels are reached however the increase in duration of cure does not quite keep pace with the increase in dose.

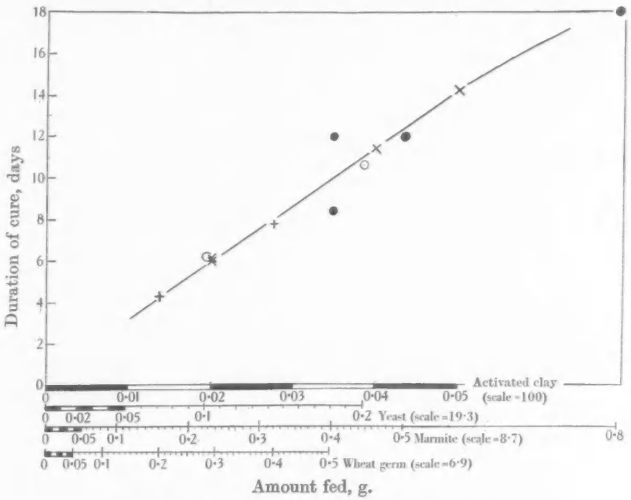

Fig. 3. Convulsion in rats: dose-effect curve.

× = Activated acid clay standard. ○ = Yeast.
● = Marmite. + = Wheat germ.

Scale divisions determined as for Fig. 2.

Degree of accuracy attainable. The relatively small degree of variation between individual animals on duplicate doses of a supplement is seen from reference to Table II. Thus at the level of 0·02 g. of activated clay, 13 out of 20 animals gave cures lasting 4 to 8 days, *i.e.* within 30 % of the median value of 6 days. Or again, with 0·4 g. of marmite 14 animals out of 22 gave a result within 20 % of the median value of 12 days, while 18 out of the 22 fell within 8–16 days, or 30 % of the median. Hence with 5 animals in a group, duplicate determinations based on the median value should certainly not differ by more than 30 %. From a practical point of view this may be taken as the "possible percentage error of the method," *i.e.* when comparing one single dose level of standard with the equivalent level of control and using 5 animals in each group. Actually, in any complete assay based on a series of such comparisons, the final error is naturally considerably less, as will be seen from Tables II and VII.

2. *Results.*

The determinations on yeast, marmite and wheat germ, taking activated clay as standard, are shown in Table II. They confirm the results given by our bradycardia method, the two sets of values being in excellent agreement.

Table II. *Cure of convulsions. Experimental results.*

Material	Dose g.	No. of tests	Days protected	Days protected, mean	Activity calculated from curve
Activated acid clay	0·01	6	(–), (–), (–), (–), (–), (–)	—	—
	0·02	13	10, 7, 6, 4, 4, 4, (20), 8, 7, 5, 7, (13), 5	6·1	—
		[7]	[5, 5, 9, 6, 11, (–), (–)]	[6]	100*
	0·04	4	13, 9, 11, 13	11·5	—
	0·05	3	17, 12, 14		
Yeast	0·05	6	(–), (–), (–), (–), (–), (–)	—	—
	0·10	9	5, 7, 5, 7, 10, 6, 6, 6, 4	6·2	20
	0·20	5	16, 8, 9, 12, 8	10·6	18·5
					19·3
Marmite	0·2	6	(–), (–), (–), (–), (–), (–)	—	—
	0·3	1	(–)	—	—
	0·4	7	10, 7, 7, 10, 6, 11, 8	8·4	7·3
		[15]	[13, 12, 14, 12, 11, 8, 12, 13, 17, 11, 12, 16, 11, 13, 22]	[12·5]	10·5
	0·5	2 [1]	[13], 12, 11	12·0	8·4
	0·8	[1]	[18]	—	—
					8·7
Wheat germ	0·1	3	(–)	—	—
	0·2	5	3, 3, 7, (–), (–)	4·3	7
	0·4	4	8, 6, 10, 7	7·8	6·8
					6·9

Figures in round brackets indicate partial cures, or abnormal response.
Figures in square brackets were determined at a separate time.
* = Standard of reference.

PART III.

Determinations by the growth rate method.

1. *Technique.*

Diet. We use a basal diet having the following composition:

Arachis oil	15
Sugar	60
"Light white casein"	20
Salt mixture	5
Alkaline autoclaved marmite after Guha and Drummond[1]	0·75 g. per rat per day
Cod-liver oil	1 drop per rat per day.

Experiments have also been carried out using rice starch in place of sucrose, and these gave identical results. In order to avoid risk of refection however we prefer to use sucrose, which has the additional advantage of being a more standard product. The "light white" variety of caseinogen is used in order to avoid any possibility of shortage of the "Coward factor".

Influence of variations in vitamin B_2 supplement. In one series of experiments 0·5 g. of autoclaved marmite was given per day in place of 0·75 g. Growth rate was consistently less. This experience emphasises the necessity, frequently overlooked, of always making comparison between animals receiving the same amount of vitamin B_2, in computing results.

[1] Made alkaline to litmus and autoclaved for 1 hour at 15 lbs.

Method of test. During the preliminary period albino rats of 50 to 70 g. weight are fed on the above diet. If smaller animals, around 40 g., are taken lower growth rates are obtained, and results are liable to be more variable. Groups should contain equal proportions of male and female, or preferably males only should be used. As soon as the animals begin to lose weight or are remaining constant, graded daily doses of unknown and standard are fed to groups of rats. The growth rate is then measured over a period of 2–3 weeks.

Calculation of results. Comparison between standard and unknown must be made over that range where small additions of the vitamin have the greatest effect on the growth rate, that is to say between 1 and 2·5 g. per day (Fig. 4). Over

Fig. 4. Growth rate method. Dose-effect curve with international standard (0·75 g. autoclaved marmite per rat per day).

this range the dose is nearly proportional to the growth rate, so that it is legitimate to calculate activity by dividing average gain in weight by dose given. It is preferable however to plot a dose-effect curve for unknown and standard. This should include at least two points for the standard on the steep part of the curve and at least one intermediate or coincident value for the unknown (or *vice versa*).

Variability of "standard reference curve." The suggestion has been made that trouble may be saved and less animals used by constructing a standard reference curve, showing the relation between dose and effect, which, it is suggested, may then be applied to any future experiment. We have found (as we reported in the memorandum to the Vitamin Conference in 1931) that this procedure is inaccurate, since the curve may vary appreciably from one experiment to another, owing to the considerable number of uncontrollable variables. Thus, in one experiment, summarised in Table III (and Fig. 4), in which five levels of standard were tested, using 4 or 5 rats, all males, in each group, the growth was found to be proportional to the dose given up to the level of about 2·5 g. per day and then fell off rapidly, whereas in the earlier experiment (Fig. 6, top), it remained proportional up to 3·5 or over. It follows that direct comparison between

Table III. *Growth responses with graded doses.*

Amount of standard fed mg. per day	Average gain in wt. g. per day	Mean gain in wt.
7	1·2, 1·7, 1·0, 1·1, 1·2	1·2
12	2·5, 2·0, 1·5, 1·9	1·9
20	2·5, 2·7, 2·4, 2·9, 2·6	2·6
25	3·1, 2·8, 2·7, 2·8, 3·1	2·8
35	3·0, 3·2, 2·6, 2·9	2·9

unknown and standard must be made in each individual experiment. This has since been accepted by Coward *et al.* [1933] in a recent paper.

Degree of accuracy attainable. Differences of ±20 % in activity can generally be readily detected by the method described. This is well illustrated by the experiment (Table IV) in which we fed supplements at 80, 100 and 120 mg. per day of marmite to groups of 4 to 5 animals only. Comparing groups of the same sex the differences in response are quite marked (*cf.* dose-response curve shown in

Table IV.

Amount of marmite fed per day mg.	Average daily gains in wt. (g.) ♂	Average daily gains in wt. (g.) ♀	Mean gains in wt. (g.) ♂	Mean gains in wt. (g.) ♀
80	0·44, 0·44	0·16, 0·12, 0·02	0·44	0·10
100	0·82, 0·82	0·24, 0·62	0·82	0·43
120	1·1, 1·3	0·45, 0·70	1·2	0·57

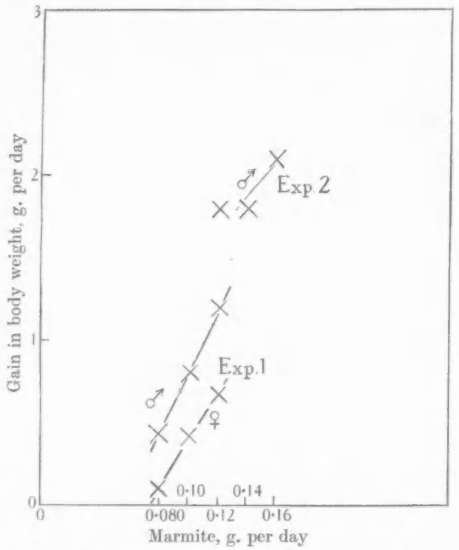

Fig. 5. Degree of accuracy of growth rate method.

Fig. 5). (In this experiment the animals grew at a somewhat slower rate than is usual for this level of marmite. This was attributed to their having started on experiment at the weight of 40 g. in place of the usual 50–70 g.)

To determine the largest individual variation in a larger group of animals and so obtain a more exact measure of the accuracy of the method, a further experiment was carried out. Doses of 160, 140 and 120 mg. of marmite respectively were fed to groups of 9, 5 and 5 animals, all males. The growth rates are given in Table V. It is seen that in the group of 9 the maximum variation is from 1·7 to

Table V. *Individual variability of animals in groups.*

Amount of marmite fed mg. per day	Average daily gains in wt. (g.)	Mean daily gain in wt. (g.)
120	0·9, 2·4, 1·8, 1·9, 1·9	1·8
140	1·8, 1·4, 1·9, 1·0, 1·8	1·8
160	2·4, 1·9, 2·3, 2·4, 2·6, 1·7, 2·0, 1·8, 1·9	2·1

2·6 g. per day, that is about 25 % either way of the median value of 2·1 g. per day. The remaining 7 animals are all within 14 % of the median. Four out of the 9 are within 10 %. In the two groups of 5, 3 or 4 animals agreed within 10 % of the median.

From the foregoing figures it may be expected that in a determination using 5 animals in a group (at a suitable level of dosage), 3 out of 5 animals should agree within 14 %; and an accuracy of this order is about the best that may reasonably be looked for. Actually in the experiment under discussion the difference between doses of 140 mg. and 120 mg. (= −14 %) was not detected when groups of 5 animals were used, but the difference between 140 and 160 mg. (= −12 %) was detected when groups of 5 and 9 animals respectively were used. On the

Table VI. *Growth method. Summary of results.*

Material	Dose (g.)	Average growth rate g. per day	Mean growth rate g. per day	Relative activities calculated from curve
1st series: 0·5 g. autoclaved marmite				
Activated clay	0·007	−1, −0·5	−0·75	
	0·014	2, 1·9, 1·7	1·87	100 (*a*)
	0·028	2·7, 2·2	2·45	
Yeast	0·04	0, 0	0	—
	0·08	1·7, 1·9, 2·6	2·07	20
	0·16	3, 2·4	2·70	21
				20·5
Marmite	0·1	−0·2, 0·5	0·15	—
2nd series: 0·75 g. autoclaved marmite				
Activated clay	0·03	4·2, 3·8	4	(103)
Yeast	0·05	1·8, 1·0	1·40	20
	0·10	3·3, 1·9	2·60	20
	0·20	3·9, 3·7, 3·3	3·63	—
				20 (*b*)
Marmite	0·07	0·75, 0·6	0·68	9·1
	0·14	2·4, 1·5, 1·6	1·83	9·6
	0·28	2·5, 3·4, 3·3	3·07	8·6
				9·1
Wheat germ	0·1	1·2, 0	0·60	6·5
	0·2	1·7, 2·3	2·00	7·5
	0·4	4·2, 3·6, 3·8	3·87	7·5
				7·2

(*a*) = Standard. (*b*) = Secondary standard.

other hand it should be pointed out that when an assay of an unknown is carried out by feeding several different levels of unknown and of standard, the final result

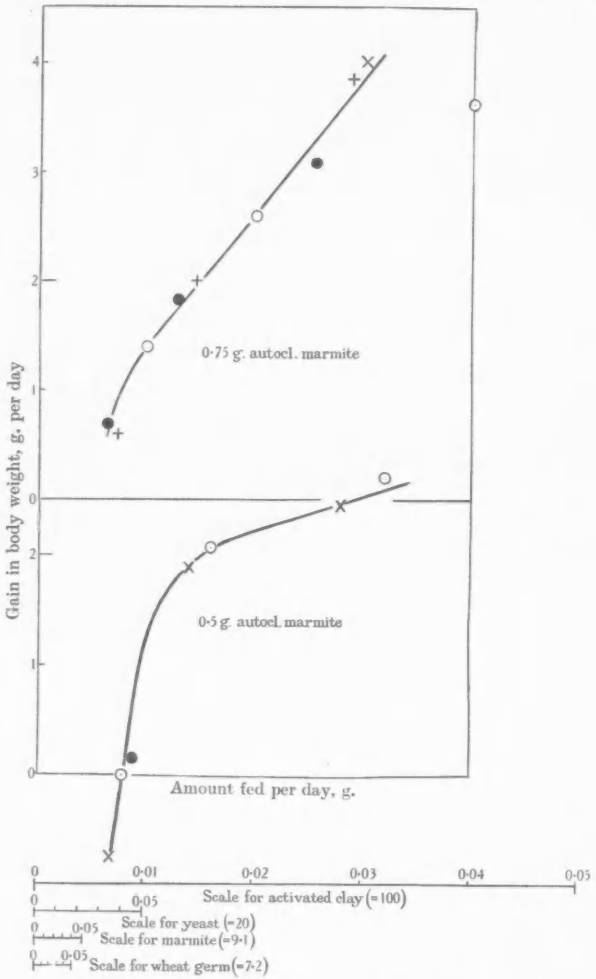

Fig. 6. Vitamin B_1 determination by growth rate method.

× = Activated clay standard. ○ = Yeast.
● = Marmite. + = Wheat germ.

Scale for abscissae determined as in Fig. 2. Note difference in response with 0·5 g. autoclaved marmite per rat per day (below) and 0·75 g. (above).

is then more accurate than just indicated, for although there is a possible error of 14–20 % in each single group, these tend to cancel each other when the final averages are determined (see Table VII).

2. *Results.*

Determinations on activated clay, yeast, marmite and wheat germ gave results virtually identical with those determined by the bradycardia method. Two determinations were carried out: in the first the yeast was compared directly with the acid clay as standard; in the second yeast was used as secondary or derived standard for determining the activity of the marmite and the wheat germ (Table VI and Fig. 6).

PART IV.

COMPARISON OF THE "BRADYCARDIA" AND OTHER METHODS OF ASSAY.

Specificity of test. The results described in Sections I, II and III show that the "bradycardia method" gave identical values with the two other methods of assay, when checked against four materials of diverse origin having activities ranging from 7 to 100. The same figures were given in a few pigeon tests which we also carried out (cure of head retractions)[1]. The comparison of these results, together with further values obtained independently in other laboratories, is given in Table VII. The values obtained by the three rat methods appear to agree within an experimental error of $\pm 5\,\%$.

Table VII. *Agreement between bradycardia and other methods.*

	Relative activities			
Method	Activated clay standard	Dried yeast	Marmite	Wheat germ
Bradycardia method	100	18·8	8·3	7
Cure of convulsions in rats	100	19·3	8·7	6·9
Curative growth tests on rats	100	20	9·1	7·2
Pigeon method	100	18	8·5	—
"Institute B" (rat growth tests)	100	(15)	9	7
"Institute C" (pigeon tests)	100	20	(11)	(14)

Values in brackets are somewhat divergent from five other concordant readings on the same material and are considered less reliable.

This very close agreement (which is confirmed by tests on vitamin B_1 concentrates and other materials) indicates that the factor measured by the bradycardia method runs parallel in its distribution with that measured by the other

[1] The basal diet used was polished rice and water. Only such pigeons as developed head retraction within 30 days were used. These amounted to about 30 % only of the total. Minimum doses were compared giving approximately the same duration of cure and corrected for the "day-dose." Our experience confirms that of other workers that the pigeon gives more erratic results than the rat owing to the greater variation in individual responses. Among the disadvantages of the pigeon method are: (1) a frequent failure to absorb the vitamin properly, especially with materials of only moderate activity, and the occurrence of a "crop bound" condition; (2) an inability to consume many types of foodstuffs of relatively low potency, but of great practical dietetic importance (which of course can be readily tested by the rat method); (3) false cures (*e.g.* as with glucose, change in temperature, *etc.*); (4) lack of economy in use—*e.g.* (*a*) the space needed to house 1 pigeon will accommodate about 10–20 rats, and (*b*) each pigeon is available for test only once in every 6 weeks, whereas with "bradycardia" or "convulsions" tests on a rat several tests per fortnight may be carried out per rat.

better known methods of assay, and it seems very probable that the same single factor is responsible for the loss in weight, for the production of the polyneuritis and for the bradycardia. Physiological arguments in favour of this view are presented in a later section of the paper (Part VI). We have found also that highly purified specimens of vitamin B_1, including the crystalline preparations of Jansen and of Windaus, retain the characteristic effect on the heart beat. At the same time we recognise that in view of the present complicated position of the vitamin B problem it would be premature to make any dogmatic claim on this point.

Advantage of the bradycardia method. The bradycardia method has many advantages over the other methods. All animals are ready for test about 4 weeks after having been placed on the deficient diet, and all consistently develop the symptom. This may be contrasted with the delay and irregularity in the production of head retractions in pigeons or convulsions in rats. Again, results are obtained very rapidly and with little trouble: only one dose has to be fed to each rat and the test is concluded in a few days, one measurement only per day being needed and taking but a few moments to carry out. Also much less material is needed than for a growth test. An additional economy is that the same animal can generally be used two or three times over. The sensitivity of the method is similar to that of the others, although it is probably slightly inferior in this respect to the growth rate method, which however is more laborious and depends on a less specific symptom. A further advantage, and a very important one from the practical standpoint, is that the heart rate method can be used for testing foodstuffs containing only slight traces of the vitamin. Neither the curative pigeon test nor the rat convulsion test is applicable to this important class of estimation, as it is impossible to get the severely ill animal to eat sufficiently large quantities of the food, as for example when the diet must consist entirely of the food in question. (The bradycardia develops at an earlier stage than the polyneuritic symptoms and while the animal is still able to eat normally.) Indeed in certain estimations of this kind, such as in comparisons of white and brown breads and of certain wheat products we have been driven back to the heart rate method as the only one available, since in growth tests on rats we were defeated by the further complication of refection so that no comparison was possible. In the heart rate method an assay can be concluded in 3 or 4 days before refection has had time to develop. It is in this type of problem, *i.e.* comparing the vitamin B activity of naturally occurring and especially farinaceous materials, that the heart rate method seems particularly useful, and we have accordingly added experimental details (Part V) of its application in certain such cases.

Practically the only drawback to the heart rate method is the original expense of purchasing the electrocardiograph. Accurate determinations are not possible with simpler types of apparatus.

PART V.

Application of bradycardia method to wheat products.

To illustrate the practical uses of the bradycardia method, its application to the determination of the vitamin B_1 content of two classes of wheat products will be described. These were (*a*) white compared with brown bread, (*b*) whole wheat grain as influenced by different soil treatments.

(a) White compared with brown bread.

We originally became interested in this problem because it had been claimed that white bread contains adequate amounts of vitamin B [Hartwell, 1924] and that the substitution of brown for white bread in a dietary experiment on rats resulted in little improvement in growth [Mottram and Hartwell, 1929].

Our attempts to compare the vitamin contents of white and brown bread by the rat growth method failed, as the rats became refected. Efforts were made to apply the rat convulsions method, but with unsatisfactory results. Determinations by the bradycardia method were then carried out, and these proved the striking difference between the two kinds of bread.

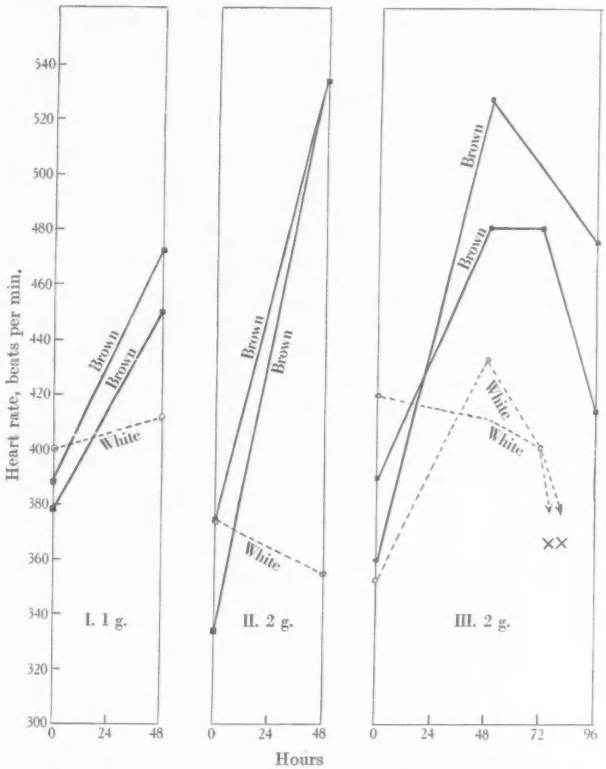

Fig. 7. Comparison of white and brown bread by the heart rate method.

Three tests were carried out. In each case the specimens of brown and white bread were purchased from a local branch of the Co-operative Society. The first test (Fig. 7, I) showed that 1 g. of brown bread was sufficient to cause a marked rise in heart rate whereas 1 g. of white bread had a very slight effect only. The difference was brought out more sharply when the brown bread was fed at a

higher level (Fig. 7, II) and a further experiment (Fig. 7, III) furnishes the basis for a rough quantitative comparison.

Conclusion. The white bread contained only insignificant amounts of vitamin B_1 compared with the brown bread. Detailed results on different types of wholemeal, brown and white and germ breads will be presented in a later paper.

(*b*) *Effect of soil treatment on vitamin value of wheat.*

This work was undertaken to test the claim that the vitamin content of seeds and grain is influenced by the nature of the manure or artificial fertiliser [McCarrison and Viswanath, 1926; Rowlands and Wilkinson, 1930].

Through the co-operation of Sir John Russell of the Rothamsted Experimental Station we were provided with specimens of whole wheat from plots treated as follows:

Plot No. 3. No manure.
2B. 14 tons of dung per acre.
5. Complete mineral manure.
7. Complete mineral manure + 412 lbs. of sulphate of ammonia per acre.
10. 412 lbs. of sulphate of ammonia only.

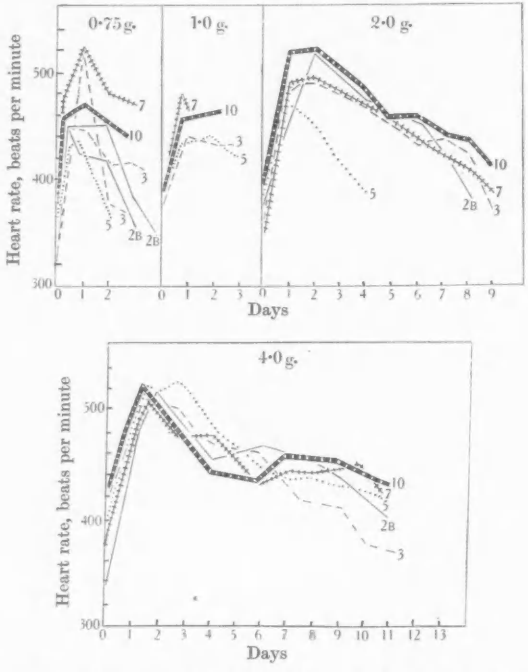

Fig. 8*a*. Vitamin B_1 value of whole meal wheat flours. Preliminary test at various levels (illustrating extent of variation of individual animals).

———— = Plot 2B. — — — = Plot 3. ····· = Plot 5.
+++++ = Plot 7. ▬▬ = Plot 10.

Although these various treatments greatly influence the chemical composition of the grain and the amount of grain produced our results (Figs. 8*a* and 8*b*) show

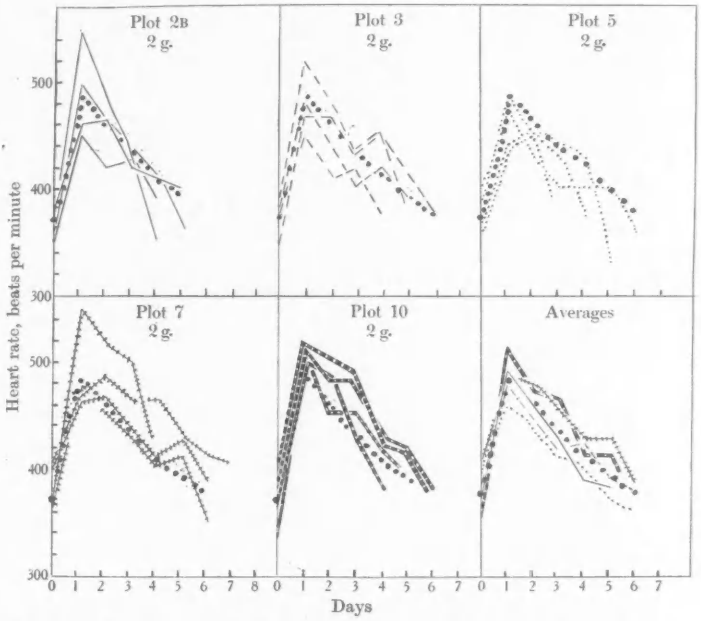

Fig. 8*b*. Comparison of vitamin B_1 activity of whole meal wheat flours, using 4 animals per group. The indication obtained in the preliminary test is confirmed, *viz.* showing the slight superiority of plots 7 and 10 and slight inferiority of plot 5, compared with 2B or 3. *Days protected:* Plots 2B and 3 = 5 days; plot 5 = 4 days; plots 7 and 10 = 6 days. *Relative activities:* Plots 2B and 3 = 100; plot 5 = 80; plots 7 and 10 = 120.

•–•–• = Comparison curve, mean of all specimens. —— = Plot 2B.
– – – = Plot 3. ····· = Plot 5. +++++++ = Plot 7. ▪▪▪▪▪ = Plot 10.

that the vitamin B_1 content when expressed per g. of whole grain is not affected to any important extent (the fuller data will be presented elsewhere). In conformity with this, growth tests on the "sharps" prepared from the grain from these same plots showed no appreciable variation in content of vitamin B complex.

PART VI.

Physiological significance of the bradycardia in vitamin B_1 deficiency.

1. *Relations between bradycardia and "polyneuritic" symptoms.*

As mentioned in Part I, the incidence of convulsions in our vitamin B_1-deficient rats was greatly increased when they were given small traces of vitamin B_1 in their diet. This symptom seems to be associated, that is, with hypovitaminosis rather than complete avitaminosis or with chronic rather than acute

vitamin B_1 deficiency. This finding confirms earlier conclusions of Sherman and Sandels [1931] and Sebrell and Elvove [1931]. (A similar relation appears to apply also to certain symptoms of vitamin B_1 deficiency in the pigeon.) Measurements of the heart rate which we have carried out suggest that this small amount of vitamin B_1 probably has the function of keeping the heart rate at a high enough value to enable the animal to survive, thus allowing the chronic symptoms of polyneuritis to develop. We find that the heart rate of a "hypovitaminotic" rat with symptoms of polyneuritic convulsions is generally around 400 beats per minute, while a rat rapidly run out on a diet completely devoid of vitamin B_1 has a much lower heart rate, about 300 beats per minute or lower, and no symptoms of "polyneuritis." In other words the biochemical lesion caused by vitamin B_1 deficiency seems to affect the heart almost immediately and may so cause sudden death from heart failure, while its poisoning action on the central nervous system proceeds more slowly.

2. *Relation to the biochemistry of vitamin B_1 action.*

In the earlier paper on this question [Drury *et al.*, 1930] the suggestion was made that the bradycardia might be correlated with the accumulation of lactic acid which is known to occur in vitamin B deficiency [Bickel, 1924; 1925; Collazo, 1923; Collazo and Morelli, 1926]. This error in lactic acid metabolism seems to be a consistent feature of vitamin B_1 deficiency, is not restricted to any one organ or type of tissue and is common to various species. Work on beri-beri patients in Japan, for example, has shown how the lactic acid tends to reach abnormally high levels, especially after exercise. Hayasaka [1930, see also Hayasaka and Inawashiro, 1928; 1930; Inawashiro and Hayasaka, 1928] has found that the recovery process after muscular exercise is more prolonged in beri-beri than in normal individuals, and, in the case of the dog, he has pointed out that in vitamin B deficiency even quite light exercise sufficed to keep the blood-lactic acid above its normal value for prolonged periods. He therefore concludes that lack of vitamin B leads to a disturbance in the resynthesis of glycogen from lactic acid. In keeping with this view he has found that when sodium lactate was injected into beri-beri patients the resynthesis to glycogen was much lower than in normal controls or cured beri-beri subjects. The accumulation of lactic acid in vitamin B_1 deficiency has been confirmed further by Collazo and Bayo [1931] and others, and Kinnersley and Peters [1930] and their co-workers have recently found a correlation between the imminence of convulsions in pigeons and the excess of lactic acid accumulating in certain parts of the brain. From what is known of the influence of lactic acid on heart rhythm [Clark *et al.*, 1932] it is to be expected that an excess will diminish the rate of beat *in vivo*, as it does in isolated preparations. Measurements of blood-lactic acid (Fig. 9) in vitamin B-deficient rats have confirmed our belief in a correlation between the lactic acid excess and the low heart rate. On the other hand it must be recognised that this does not

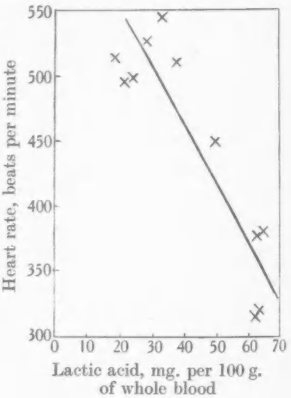

Fig. 9. Correlation between fall in heart rate and rise in lactic acid content of blood in vitamin B_1 deficiency.

prove that the correlation is a direct causative one. Attempts to influence the rate by the administration of large doses of sodium lactate have been unsuccessful.

Experimental. It was found necessary to use about 3 rats in order to obtain enough blood for each lactic acid estimation. The blood samples were obtained by decapitating the rats with scissors over a weighed basin of 20 % trichloroacetic acid. The blood was allowed to drain from the animals for about half a minute, the basin was then again weighed and the amount of blood obtained calculated from the difference in the weighings. The amount of blood used was usually between 6–10 g. this giving a reading of approximately 1–3 ml. of $N/100$ I in the final titration. When sufficient blood had been obtained the trichloroacetic acid mixture was made up to a definite volume so as to give a final concentration of about 5 % trichloroacetic acid. This solution was then filtered and precipitated with the copper-lime reagent to remove carbohydrates and the lactic acid estimation carried out according to the method of Friedemann *et al.* [1927].

As will be seen from Fig. 1 the heart rate of a normal rat is between 500 and 600 beats per minute, and the lactic acid content is about 30 mg. per 100 g. of blood. When the rats become deficient in vitamin B_1 the heart rate drops to 300–350 beats per minute and the lactic acid content rises to about 70 mg. per 100 g.

3. *Vitamin B_1 as oxidative coenzyme.*

The existing evidence warrants the suggestion that the physiological rôle of vitamin B_1 is to intervene at some stage in that complex cycle of reactions in carbohydrate metabolism involving the formation and oxidation of lactic acid, and in a capacity corresponding with that of coenzyme. Banga and Szent-Györgyi [1932] announced the isolation of a coenzyme for lactic acid dehydrogenase. One of us has drawn attention elsewhere [Harris, 1934] to the fact that a number of salient properties of the coenzyme bear a resemblance, superficially at least, to those of vitamin B_1—*e.g.* it is needed for the normal functioning of the heart muscle (it was from this source that it was isolated), and it is concerned in the removal and oxidation of lactic acid. In this connection also its suggested relationship to adenine, or adenylic acid, which is known to be of significance for the physiology of the heart beat, is of further interest.

It seems certain of course that vitamin B_1 itself cannot be identical with Banga and Szent-Györgyi's coenzyme (*cf.* Boyland, 1933); nevertheless there appears to be a close interconnection between the two classes of substances and their physiological rôles in the related and interdependent series of reactions which they control. The nature of the lactic acid dehydrogenase system is discussed further in the following paper [Birch and Mann, 1934].

Summary.

Part I.

The technique of estimating vitamin B_1 by the heart rate method which has been in use in this laboratory for several years as a standard method is described. Single doses of the unknown are fed to rats suffering from vitamin B_1 deficiency and the heart rate measured at daily intervals for a few days afterwards. The magnitude and duration of the effect are proportional to the amount of vitamin so given. The accuracy of the method was checked by tests on four substances chosen for investigation by the Sub-committee on Vitamin B_1 Standardisation, *viz.* activated acid clay standard, dried yeast, marmite and wheat germ. These were assayed by separate control tests by the independent methods described in Parts II and III.

Part II.

A method of carrying out vitamin B_1 determinations by curative tests on polyneuritic convulsions in rats is described. A feature of this method is the close agreement in response between animals receiving duplicate doses.

Part III.

Results with a growth rate method are also given and the conditions defined for the most accurate results. The number of variables differs too much to permit the use of a standard dose-effect reference curve—as for example with the amount of autoclaved marmite given as the source of vitamin B_2. Under the conditions described differences of ± 20 % in activity are readily detected, and 3 out of 5 animals at least in a group fall within ± 15 % of the median value while the values reached in the assays by any of the three rat methods appeared to vary within an experimental error of about ± 5 %.

Part IV.

Results by the heart rate method correspond with those given by the other methods, the comparative values found, taking activated clay standard as 100, being yeast 18·8, 19·3, 20, 18; marmite 8·3, 8·7, 9·1, 8·5; wheat germ 7, 6·9, 7·2, by the bradycardia, convulsions, growth rate and pigeon (head retraction) methods respectively. Among the advantages of the heart rate method are its convenience, rapidity, simplicity and economy, and the fact that it can be readily used for determining foodstuffs containing only small amounts of vitamin B_1 which are beyond the scope of the curative pigeon or rat (convulsions) method, and—when they contain starch and give rise to refection—the rat growth test.

Part V.

As an example of the use of the heart rate method tests are described on white and brown breads and other wheat products. These could not be readily estimated by the pigeon or rat curative methods, and growth tests on rats were vitiated by the occurrence of refection.

Part VI.

A rat given no vitamin B_1 and dying from acute avitaminosis has a very low heart rate (*e.g.* 300 per min.) and no polyneuritic convulsions. A rat given slight traces of vitamin B_1 and developing chronic hypovitaminosis has a less severe bradycardia (*e.g.* heart rate 400) but convulsions. It is suggested that the traces of vitamin B_1 act by keeping the heart rate at a high enough level to enable the animal to survive and so permit the more slowly developing chronic symptom to develop.

The earlier suggestion is confirmed that the bradycardia is correlated with the accumulation of lactic acid in the vitamin B_1-deficient animal. Vitamin B_1 appears to have the property of a coenzyme-like substance intervening in the chain of carbohydrate oxidation reactions.

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LXXXVI. THE ACTIVATION OF LACTIC DEHYDROGENASE AND ITS RELATION TO THE RÔLE OF VITAMIN B_1.

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The necessity of a coenzyme for the oxidation of lactic acid by the lactic dehydrogenase of animal tissues has been shown by Szent-Györgyi [1925]. The preparation of a concentrate of this substance from mammalian heart tissue has been described by Szent-Györgyi and Vargha [1932]. Recently Banga and Szent-Györgyi [1933] have further purified the concentrate by precipitation with phosphotungstic acid and picric acid and have obtained the coenzyme as a semi-crystalline picrate. By investigation of the products of hydrolysis of the coenzyme they concluded that it was a monophosphate of a monoadenylnucleotide not identical with muscle adenylic acid or adenylpyrophosphate, but possibly identical with cozymase.

This coenzyme is of considerable interest owing to the importance of lactic acid as an intermediary in animal metabolism. In certain pathological conditions where accumulation of lactic acid is observed, it appears possible that such an accumulation may be due to a deficiency of the coenzyme. Such an accumulation occurs as a symptom of vitamin B_1 deficiency and in this case there is additional evidence which suggests the possibility of a connection between vitamin B_1 activity and the coenzyme activity.

Kinnersley and Peters [1929; 1930] showed that brain tissue of polyneuritic pigeons had an abnormally high lactic acid content. Fisher [1931] has shown that there is a general defect in lactic acid metabolism in the polyneuritic pigeon as a whole, in addition to the defect in brain described by Kinnersley and Peters. This defect in lactic acid metabolism in vitamin B_1 deficiency is not confined to pigeons. According to Japanese workers, *e.g.* Hayasaka [1930], lactic acid accumulates in the body and reaches abnormally high levels in patients suffering from beri-beri.

Further, a relation between vitamin B_1 and lactic dehydrogenase coenzyme is suggested from the work of Meiklejohn *et al.* [1932] and Passmore *et al.* [1933], who showed that the cerebrum of the brain of polyneuritic pigeons when minced and mixed with lactic acid showed a low oxygen uptake compared with normal brain tissue. The addition of vitamin B_1 concentrate increased the *in vitro* respiration of the polyneuritic brain tissue, and it was assumed that the oxidation affected in this way was that of lactic acid.

Unfortunately, from the recent work of Meiklejohn [1933] it appears that the amount of lactic acid removed during the oxidation is not influenced by the presence of the vitamin. A similar observation has been made by Hahn and Fischbach [1929] using ox muscle. They find that with washed ox muscle, the

[1] Beit Memorial Research Fellow.

renewed oxygen uptake in presence of "Kochsaft" is not due to oxidation of the added lactic acid, for although the amount of respiration may be restored to 90 % of its original value by adding the "Kochsaft," the lactic acid content remains unaltered. On the other hand, while avitaminous brain tissue shows a low oxygen uptake with pyruvic acid similar to that with lactic acid, according to the conclusions of Meiklejohn *et al.* [1933] in this case the addition of vitamin B_1 is without effect. However, on consideration of the results of Peters and his collaborators it appears that this conclusion is somewhat unjustified. The difference in the rate of oxygen uptake between normal and avitaminous brain tissue in presence of lactate is small. Hence the increase obtained on addition of vitamin B_1 to the avitaminous brain is almost sufficient to raise the rate of oxygen uptake to that of normal brain tissue + lactate. On the other hand the difference in the rate of oxygen uptake in the case of normal and avitaminous brain with pyruvate is much greater than in the case of lactate. Hence in this case while addition of vitamin B_1 causes an increase of the same order as in the case of lactate, this addition is insufficient to raise the rate to normal. In the case of lactate and avitaminous brain, using the cerebrum the increase in oxygen uptake produced by addition of vitamin B_1 is 28 %, using the optic lobes 22 %. In the case of pyruvate with the cerebrum the increase in oxygen uptake produced by addition of vitamin B_1 is 20 %, and with the optic lobes 40 %. If the increase in the presence of lactate is to be regarded as significant it appears that the case of pyruvate should not be ignored merely because it does not raise the rate of oxygen uptake to normal. Furthermore since the difference in the rate of oxygen uptake between normal and avitaminous brain with pyruvate is greater than with lactate, it appears that deficiency of vitamin B_1 produces actually a greater effect on the mechanism of pyruvate oxidation than on that of lactate oxidation. Peters explains his conclusion by assuming that the increase obtained with pyruvate is due to the effect of the vitamin on the residual lactic present in the brain tissue.

The question of the possible identity of vitamin B_1 and lactic dehydrogenase coenzyme has been investigated by Boyland [1933]. As vitamin B_1 is a sulphur-containing compound and the coenzyme contains no sulphur, it is difficult to imagine how the two can be identical. Further arguments point in the same direction, for example Szent-Györgyi precipitates his coenzyme with mercuric nitrate, followed by precipitation from aqueous solution with acetone. By neither of these reagents would vitamin B_1 be precipitated. Of course it still remains possible that vitamin B_1 might exist in the coenzyme in some such form as the base of a nucleotide which occurred as a small impurity in Szent-Györgyi's preparation, but Boyland's experiments do not allow for this.

Boyland concluded that crude coenzyme preparations possessed only a slight vitamin B_1 activity as determined by rat feeding experiments, and that while crude vitamin B_1 concentrates showed activity as coenzyme for heart-lactic dehydrogenase, purified preparations did not possess this activity.

Table I shows the results obtained by Boyland in testing the activity of his coenzyme preparation by the methylene blue technique.

Table I.

Lactic dehydrogenase coenzyme solution 10 mg. per ml.	*M*/10 lactate ml.	Reduction time in minutes
0·0	1·0	180
0·1	1·0	47
0·2	1·0	27
0·4	1·0	12½
0·4	0·0	150

Since in the above experiments doubling the concentration of the coenzyme doubles the velocity of the reaction, it appears doubtful whether 0·4 ml. of the coenzyme solution is sufficient to produce maximum activation of the enzyme. Meiklejohn *et al.* [1932] found in their experiments with avitaminous brain tissue and lactate that it was necessary to add only 1/10 of a pigeon dose of vitamin B_1 to each Barcroft cup to obtain the maximum increase in oxygen uptake. Thus in the case of Boyland's results, if it is assumed that 0·4 ml. of the coenzyme solution produces maximum activation of the enzyme then this amount should be equivalent to 1/10 of a pigeon dose. Boyland tested the vitamin B_1 activity of his preparation by rat feeding experiments. He found, as would be expected, that 0·1 ml. and 0·2 ml. showed no activity, while 2 ml. showed some activity, but less than one pigeon dose. From the above calculation, 2 ml. should only be equivalent to half a pigeon dose or less.

Thus Boyland's conclusion that lactic dehydrogenase coenzyme possesses little vitamin B_1 activity appears unjustified and in spite of his work it still seems possible that there may be a close interrelation between vitamin B_1 and the coenzyme activity. The work to be described was started with the view of investigating this possibility.

Preparation of the enzyme.

During the course of this work three different methods have been employed for the preparation of lactic dehydrogenase from heart muscle. The methods so employed may be divided into two sections according to the properties of the enzyme preparation obtained.

Section I. Szent-Györgyi and Vargha's method [1932]. 40 g. pig heart muscle freed from fat and connective tissue are finely chopped and shaken with 800 ml. of distilled water at room temperature for 10 minutes; after squeezing through linen the residue is shaken with another 800 ml. of distilled water for a further 10 minutes. After squeezing through linen, the residue is placed in a mortar and frozen by surrounding it with an ice and salt freezing mixture. The tissue is then ground up and mixed with 120 ml. of $M/15$ phosphate buffer at p_H 7·2.

Section II. (*a*) *Boyland's method* [1933]. 20 g. of well minced pig's heart are ground up with 250 ml. of ice-cold water and pressed through linen. The residue is washed in this way six times, pressed and then frozen. While frozen it is minced three times to break up the cells and then suspended in 100 ml. $M/100$ phosphate buffer at p_H 7·4.

(*b*) *Birch and Mann's method.* In this method the enzyme is extracted from the tissue. The method is identical in its initial stages with that of Szent-Györgyi. It is essential that the frozen tissue should be thoroughly ground up so as to break up the cells as far as possible. The pounded tissue is suspended in phosphate buffer and allowed to stand for 2–3 hours at room temperature. The suspension is then squeezed through linen, and the liquid obtained is fully saturated with ammonium sulphate. The precipitate is filtered off and dried in a vacuum-desiccator. The yield from 100 g. of tissue is about 7 g. This enzyme preparation shows little loss of activity after several weeks if it is left in the dry form in a vacuum-desiccator. It may be further purified by dissolving in phosphate buffer, centrifuging off the insoluble residue and then reprecipitating with ammonium sulphate.

The differentiation of the methods of preparation is based on the amount of washing which the tissue undergoes. Szent-Györgyi's enzyme preparation consists of comparatively unwashed tissue, while by Boyland's method the tissue is

very thoroughly washed. In our own method the enzyme is further purified by extraction from the tissue and by precipitation with ammonium sulphate. This difference in the degree of washing of the tissue is accompanied by a fundamental difference in the properties of the resultant enzyme preparations.

Preparation of the coenzyme.

At the beginning of this work the method of Szent-Györgyi and Vargha [1932] was used for the preparation of the coenzyme from bullock's heart and Boyland's enzyme was used as a means of testing the activity of the coenzyme. The yield of the coenzyme is small—about 0·7 g. of the crude concentrate from 5 kg. of heart, and in the subsequent purification by phosphotungstic acid most of the activity was lost. In place of the phosphotungstic precipitation other methods were used for the purification of the crude coenzyme, and in some cases it was apparently separated into two fractions, either of which separately showed little coenzyme activity but together gave good activation of the enzyme. An example of this, where precipitation with silver nitrate was used as the means of fractionation is shown in Table II.

Solution I. The crude coenzyme solution is precipitated at p_H 1·5 with $AgNO_3$. The precipitate is decomposed with H_2S, evaporated to a small volume and precipitated by the addition of 10 volumes of acetone. This precipitate dissolved in water forms solution I.

Solution II. The filtrate from the above acid silver precipitate is brought to p_H 6 with ammonia and again precipitated with $AgNO_3$. The filtrate is treated with H_2S, evaporated and precipitated with acetone. The acetone filtrate is evaporated until all the acetone is removed.

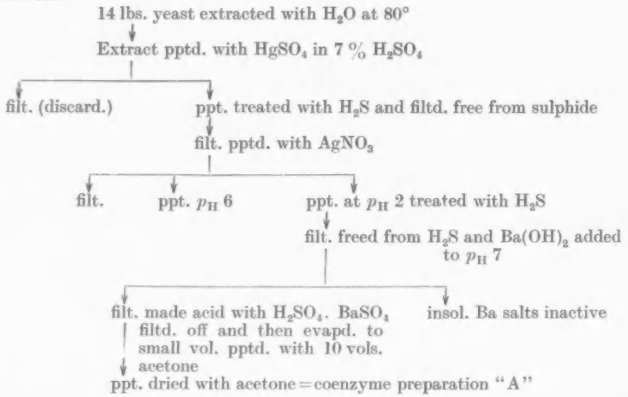
Using these two solutions with Boyland's enzyme the following results were obtained by the Thunberg technique of methylene blue reduction. The total volume in each tube was 3 ml. In general 1 ml. of enzyme was used and 1 ml. of 1 in 5000 methylene blue, together with phosphate buffer at p_H 7·4. In some cases when the enzyme was found to be weak the methylene blue was cut down to 0·5 or 0·25 ml.

Table II.

	Reduction time (mins.)
0·25 ml. of (I) + enzyme + lactate + MB	80
1·0 ml. of (II) + enzyme + lactate + MB	120
0·25 ml. of (I) + 1·0 ml. of (II) + enzyme + lactate + MB	10
0·5 ml. of (I) + 0·5 ml. of (II) + enzyme + MB (no lactate)	68

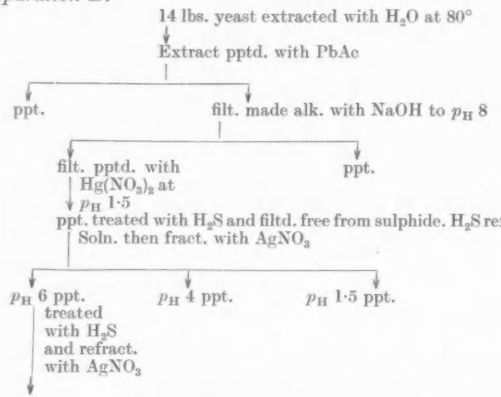
Attempts to separate these two factors from heart completely were unsuccessful, though results such as those shown in the above table were sometimes obtained. One of the causes of this failure was the minute amounts of the substances with which we were dealing. It was found that yeast contained considerable amounts of the coenzyme. Since yeast is more readily obtainable and easier to work up on a large scale than heart tissue, it formed the source of most of our subsequent preparations of coenzyme. Even using yeast it has so far proved impossible to effect a complete separation of the two factors. One factor, which is probably identical with Szent-Györgyi's coenzyme is readily obtained more or less free from the other. The method of obtaining this is shown in the following scheme.

Preparation A.


14 lbs. yeast extracted with H_2O at 80°
Extract pptd. with $HgSO_4$ in 7 % H_2SO_4
filt. (discard.)
ppt. treated with H_2S and filtd. free from sulphide
filt. pptd. with $AgNO_3$
filt.
ppt. p_H 6
ppt. at p_H 2 treated with H_2S
filt. freed from H_2S and $Ba(OH)_2$ added to p_H 7
filt. made acid with H_2SO_4. $BaSO_4$ filtd. off and then evapd. to small vol. pptd. with 10 vols. acetone
insol. Ba salts inactive
ppt. dried with acetone = coenzyme preparation "A"

Attempts to obtain the second factor free from the first have up to the present been unsuccessful. The best preparations so far consist of a mixture of the two factors. The method of preparation of this mixture is shown in the following scheme.

Preparation B.


14 lbs. yeast extracted with H_2O at 80°
Extract pptd. with PbAc
ppt.
filt. made alk. with NaOH to p_H 8
filt. pptd. with $Hg(NO_3)_2$ at p_H 1·5
ppt.
ppt. treated with H_2S and filtd. free from sulphide. H_2S removed by evap. *in vacuo*.
Soln. then fract. with $AgNO_3$
p_H 6 ppt. treated with H_2S and refract. with $AgNO_3$
p_H 4 ppt.
p_H 1·5 ppt.

Most of the activity is found in the silver ppt. at p_H 6 while some activity is also found in the p_H 1·5 ppt. These ppts. are again fractionated with silver nitrate. The silver fractionation does not give clear cut results. The majority of the activity usually comes down at p_H 6, but often some comes down at p_H 1·5. These solutions obtained after refractionation with silver are called "coenzyme preparation B" in the subsequent experiments.

Using preparation "A" in the Thunberg technique of methylene blue reduction only small amounts are necessary to produce maximum activation (0·5 to 2 mg.; the actual amount seems to vary with the enzyme used); the activation produced is small. With preparation "B" relatively large amounts are often necessary but the activation produced is 5–8 times as great when using

the pure enzyme. It is seen from the schemes of preparation shown that the first factor "A" is precipitated by $HgSO_4$ and by acetone from aqueous solution. The second factor does not seem to follow these reactions, but if the $HgSO_4$ filtrate or the acetone filtrate is examined for coenzyme activity it is usually found to possess only slight activity. In some cases by mixing the two solutions reactivation has been obtained as is shown in Table II, but more often than not the mixture is found to be inactive. Thus it appears that the second factor is unstable except when in the presence of the first, and the best preparation obtainable is a mixture of the two, containing a greater percentage of the second factor.

Further evidence for the existence of two factors.

In addition to the results shown in Table I other evidence has been found which suggests that two factors are necessary. This has been obtained by comparing the maximum activation produced by coenzyme preparations "A" and "B" with each type of enzyme.

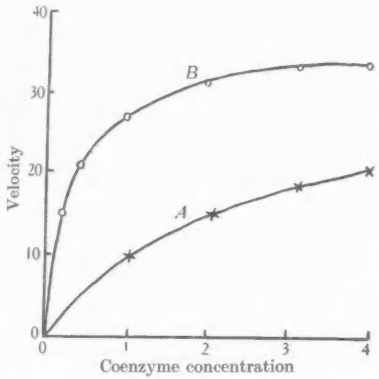


Fig. 1.

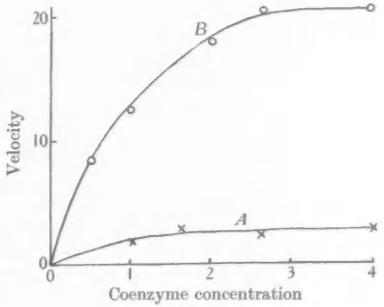


Fig. 2.

Fig. 1. Comparison of the two coenzyme preparations "A" and "B" using the unpurified enzyme at p_H 7·2. Abscissa = relative amounts of coenzyme added; ordinate = $1/T$.

In this experiment 1 ml. of Szent-Györgyi's enzyme was used with 1 ml. of 1 in 5000 methylene blue, the lactate concentration being $M/10$. The minimum reduction time with coenzyme preparation "B" was 3 minutes. The minimum reduction time with coenzyme preparation "A" was 5 minutes. The blanks were enzyme + lactate + MB = 135 minutes, enzyme + maximum amount of coenzyme "B" + MB = 43 minutes, enzyme + coenzyme "A" + MB = 150 minutes.

Fig. 2. Comparison of coenzyme preparations "A" and "B" with the purified enzyme at p_H 7·2. This enzyme was twice precipitated with ammonium sulphate. Starting with 1 g. of the original precipitate the final volume was made up to 20 ml., and ½ ml. of the solution was used. The minimum reduction time with coenzyme preparation "B" was 4¾ minutes. The minimum with coenzyme preparation "A" 31½ minutes. The blanks were enzyme + lactate + MB = 6 hours + ; enzyme + coenzyme "A" + MB = 6 hours; enzyme + coenzyme "B" + MB = 51 minutes.

Fig. 1 shows curves obtained using Szent-Györgyi's enzyme; curve A with increasing amounts of coenzyme "A," curve B with coenzyme "B." With this unpurified enzyme coenzyme "A" gave an activation which was almost two-thirds of that obtained with "B."

When a more purified enzyme was used with increasing amounts of the coenzyme results were obtained which are shown in Fig. 2. Here it is seen that

maximum activation with coenzyme "A" was only one-seventh of that given by coenzyme "B."

Thus using an enzyme consisting of practically unwashed tissue both coenzymes show similar activity. Using a purified enzyme there is a marked difference in the activation produced by the coenzyme preparations "A" and

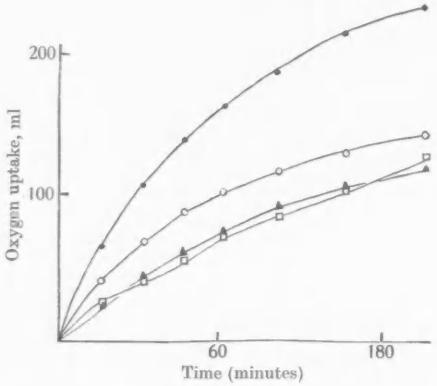


Fig. 3.

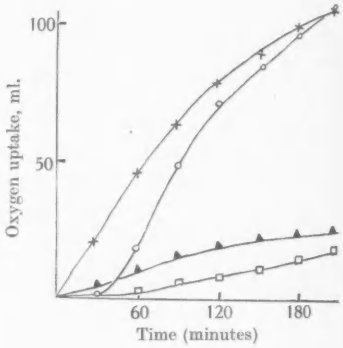


Fig. 4.

Fig. 3. Rate of oxygen uptake using Szent-Györgyi's enzyme at p_H 7·2.

▲, {Right-hand cup contained enzyme + lactate + coenzyme "A"
{Left-hand cup contained enzyme + lactate

□, {Right-hand cup contained enzyme + lactate + coenzyme "A"
{Left-hand cup contained enzyme + coenzyme "A"

●, {Right-hand cup contained enzyme + lactate + coenzyme "B"
{Left-hand cup contained enzyme + lactate

○, {Right-hand cup contained enzyme + lactate + coenzyme "B"
{Left-hand cup contained enzyme + coenzyme "B"

2 ml. of enzyme were used, the lactate concentration being $M/20$. The amount of coenzyme used was that which was found necessary to give maximum activation in 3 ml. when using the methylene blue technique.

The difference between the two curves obtained with coenzyme "B" is presumably due to some blank in the coenzyme preparation, which is accounted for in ○ where it is balanced against enzyme + coenzyme.

Fig. 4. Oxygen uptake using ammonium sulphate enzyme at p_H 7·2.

×, {Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
{Left-hand cup contained enzyme + lactate

○, {Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
{Left-hand cup contained enzyme + coenzyme preparation "B"

▲, {Right-hand cup contained enzyme + lactate + coenzyme preparation "A"
{Left-hand cup contained enzyme + lactate

□, {Right-hand cup contained enzyme + lactate + coenzyme preparation "A"
{Left-hand cup contained enzyme + coenzyme preparation "A"

Ammonium sulphate coenzyme 1 g. made up to 18 ml. in phosphate buffer, then 2 ml. used. The lactate and coenzyme concentrations were the same as in the previous experiment.

"B." With a purified enzyme preparation "A" is practically inactive. Thus preparation "B" contains an additional factor necessary for the activation of lactic dehydrogenase. This factor is present in Szent-Györgyi's crude enzyme, and thus coenzyme preparation "A" shows activity. On purification of the enzyme

however this factor is removed and preparation "A" becomes inactive with this enzyme, while preparation "B" still remains active.

This explanation is again supported by similar results obtained by using the Barcroft technique for the measurement of the rate of oxygen uptake. The results using Szent-Györgyi's enzyme are shown in Fig. 3. The amount of coenzyme added was the same as that which was required to give maximum activation in 3 ml. when using the Thunberg technique. It is seen that coenzyme "A" gives a rate of oxygen uptake which is about half that given by using coenzyme "B."

When using the purified enzyme curves were obtained which are shown in Fig. 4. In this experiment the rate of oxygen uptake with coenzyme "A" is only one-fifth of the rate obtained by using coenzyme "B."

Thus the results obtained by the Barcroft technique support those obtained by the Thunberg technique and indicate that two factors are necessary for the activation of lactic dehydrogenase. In the Barcroft experiment just described with the ammonium sulphate enzyme the partially purified enzyme was used, not that purified by a second precipitation with $(NH_4)_2SO_4$. This was because by further purification of the enzyme in this manner we appear to remove a factor necessary for oxygen uptake by the system. This is shown in Fig. 5 where we have used the twice precipitated enzyme with coenzyme preparation "B" with and without methylene blue.

It is seen that the addition of methylene blue enables the system to take up oxygen about twice as fast as when methylene blue is absent. The system has not yet been obtained in such a state that it gave no oxygen uptake without methylene blue but these results seem to indicate that lactic dehydrogenase with its coenzymes is unable to take up oxygen by itself. Apparently it needs some system such as the cytochrome system to enable it to take up oxygen. The addition of methylene blue to systems containing the cruder enzyme has no effect on the oxygen uptake.

The possible identity of these coenzymes with cozymase.

In his latest paper Szent-Györgyi suggests that his factor is possibly identical with cozymase. During the course of this work two preparations of cozymase have been made according to the method of Euler and Myrbach [1928] and it was found that both were completely inactive as coenzyme for lactic dehydrogenase, while they showed a high activity as coenzyme for glucose dehydrogenase [Mann, 1932]. The estimation of the preparations as cozymase is at present being carried out and until this is done it cannot be claimed definitely that cozymase is inactive as the coenzyme. But from the results with one cozymase preparation which are shown in Table III and from the fact that the method of preparation of cozymase differs markedly from the preparation of lactic coenzyme the conclusion that they are not identical is fairly safe.

Table III. *Activity of cozymase as lactic coenzyme. Cozymase prepared according to Euler and Myrbach [1928]. Szent-Györgyi's crude enzyme used.*

	R.T.
0·1 mg. cozymase + enzyme + lactate + MB	4 hrs. +
0·5 mg. ,,	4 hrs. +
2·5 mg. ,,	4 hrs.
4·0 mg. ,,	4 hrs.
Blank with lactate + enzyme + MB	4 hrs. +
1·6 mg. P.T.A. heart coenzyme + enzyme + lactate + MB	4¼ mins.

Inhibition by pyruvic acid.

It is difficult to understand why for a simple reaction such as the conversion of lactic acid into pyruvic acid two coenzymes should be necessary. Is the second factor a coenzyme of this reaction or of some subsequent one? Using the Thunberg technique with purified enzyme and coenzyme preparation "A" or, as we

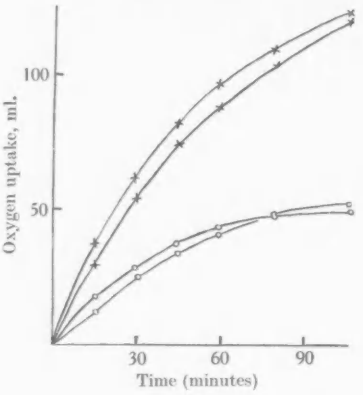


Fig. 5.

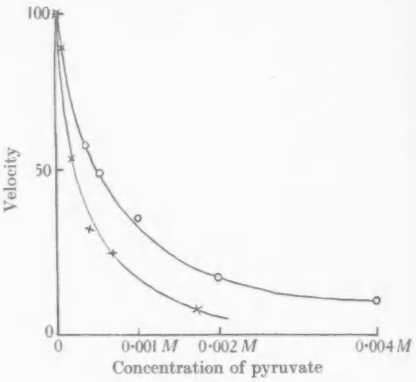


Fig. 6.

Fig. 5. The difference between the rate of oxygen uptake when methylene blue is added to the purified lactic dehydrogenase system.

o, { Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
Left-hand cup contained 3 ml. phosphate buffer.

×, { Right-hand cup contained enzyme + lactate + coenzyme preparation "B" + MB
Left-hand cup contained 3 ml. phosphate buffer.

The enzyme was prepared by dissolving the 1st ammonium sulphate precipitate in phosphate buffer, centrifuging off the insoluble matter and then reprecipitating with ammonium sulphate. 0·5 g. of this precipitate was made up to 8 ml. and 1 ml. was used in each cup.

Fig. 6. Inhibition of the activity of lactic dehydrogenase by small amounts of pyruvic acid.

o, Coenzyme preparation "B"
×, Coenzyme preparation "A"

Ammonium sulphate-precipitated enzyme used: 1 g. made up to 28 ml. 0·5 ml. of this solution was used with coenzyme preparation "B." The minimum reduction time (*i.e.* without pyruvate) was $2\frac{3}{4}$ minutes and the maximum when pyruvate was added 28 minutes. The blank on coenzyme + enzyme alone was 68 minutes. 1 ml. of the enzyme solution was used with coenzyme preparation "A" and a minimum reduction time of $6\frac{3}{4}$ minutes was obtained. The maximum when pyruvate was added was 98 minutes.

The concentration of lactate was $M/10$, and 1 ml. of 1 in 5000 methylene blue was used throughout. The pyruvic acid was purified according to the method of Case [1932], and was added as the sodium salt.

believe, Szent-Györgyi's coenzyme, it was often found that for a short period reduction of methylene blue proceeded quite rapidly, after which further reduction was very slow. It therefore appeared that some substance which was being formed was inhibiting the reaction and it appeared probable that this substance might be pyruvic acid. This view was supported by results obtained from the effect of added pyruvic acid on the velocity of reduction of methylene blue in presence of $M/10$ lactate. The results are shown in Fig. 6.

It is seen from Fig. 6 that a minute concentration of pyruvate inhibited the reaction, using coenzyme "A" with the ammonium sulphate enzyme. This may

afford an explanation of the slowing off in the speed of reduction of M.B. when this coenzyme was used, as immediately a trace of pyruvic acid was formed it inhibited the reaction.

Using coenzyme "B" a much greater concentration of pyruvate was necessary to give the same percentage inhibition. It is suggested as an explanation of this that in the case of coenzyme "B" some factor was present which enabled the removal of pyruvic acid. In the case of coenzyme "A" this factor was missing.

The second factor present in the coenzyme "B" would therefore be a coenzyme for removal of pyruvic acid.

One would naturally suppose that this removal would be carried out by oxidation, but if this is so it seems that the oxidation is not accomplished by pyruvic dehydrogenase since pyruvate without lactate in the presence of enzyme and coenzyme hardly reduced methylene blue at all.

Fig. 6 shows that an apparent equilibrium or maximum inhibition with pyruvate was obtained with a pyruvate concentration between 0·002 and 0·004 *M*. This differs from the results of Bernheim [1928] who found that this "apparent equilibrium" was obtained between 0·033 and 0·066 *M* concentration of pyruvate. This was presumably due to the presence of our second factor in his enzyme. It must be borne in mind however that it has not yet been shown that the soluble lactic dehydrogenase from yeast needs a coenzyme but, as the coenzyme for heart muscle-lactic dehydrogenase occurs in relatively large amounts in yeast, it is conceivable that it does. This matter is at present under investigation.

Activity of the coenzyme preparations "A" and "B" as vitamin B_1.

The difference between the coenzymes "A" and "B" as activators of lactic dehydrogenase is accompanied by a difference in their action on vitamin B_1-deficient rats. Rats as the experimental animals have been used and the heart rate method as the method of estimating vitamin B_1 activity. Normal rats (heart rate 500–550); vitamin B_1-deficient rats (heart rate 300–350). Injection of vitamin B_1 intraperitoneally causes the heart rate of vitamin B_1-deficient rats to return to normal, usually in about 6 hours.

Injection of coenzyme "A" into vitamin B_1-deficient rats caused an almost instantaneous drop in heart rate. This supports Szent-Györgyi's opinion that his coenzyme is an adenylic acid, as Drury and Szent-Györgyi [1929] have shown that certain adenine compounds, notably adenosine and adenylic acid, caused bradycardia in normal rats when injected. Using coenzyme "A" this drop in the heart rate lasted for 1–2 hours depending on the amount injected and then the rate returned to nearly its original value. It showed no rise above its original value and therefore no vitamin B activity.

In these experiments that amount of coenzyme which produced maximum activation in a total volume of 3 ml. in a M.B. tube was called one dose.

Using the coenzyme "B," if 9 doses were injected a similar drop in heart rate was produced to that obtained with coenzyme "A," showing that the adenine compound was again present. The heart rate returned to its original value after 1–2 hours, and tended to rise slightly above the original value, but this slight rise was only maintained for a few hours. Using half the amount the drop was less, and the subsequent rise was quicker, the heart rate returning to its original value in about half an hour and continuing to rise above its original value during the next 2 or 3 hours. This seemed to show some definite curative effect.

Using $2\frac{1}{2}$ doses, while the initial drop in heart rate was smaller and recovery to original rate more rapid, the subsequent rise in heart rate was much smaller than with $4\frac{1}{2}$ doses. These results are shown in Fig. 7.

The curative effect of the coenzyme "B" was limited to a few hours whereas injection of vitamin B_1 with or without the coenzyme gave a curative effect lasting for several days.

This was shown in Fig. 8 where 9 doses of coenzyme "B" and vitamin B_1 were injected simultaneously.

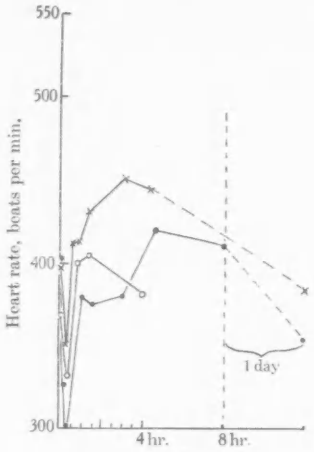


Fig. 7.

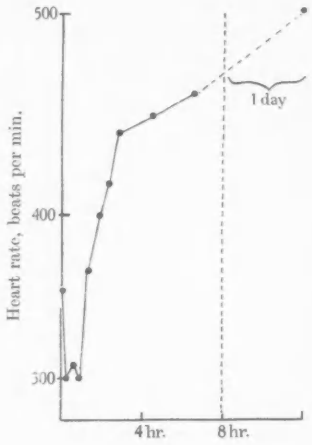


Fig. 8.

Fig. 7. Effect of coenzyme preparation "B" on the heart rate of vitamin B_1-deficient rats. One dose is the amount of coenzyme necessary to give maximum activation of the enzyme in 3 ml.

•, 9 doses injected intraperitoneally
×, $4\frac{1}{2}$ doses injected intraperitoneally
⊙, $2\frac{1}{4}$ doses injected intraperitoneally

The coenzyme preparation used in this experiment was prepared by a slight modification of scheme B. The original yeast extract was first of all treated with acid clay at p_H 4·5 in order to adsorb as much vitamin B_1 as possible. The acid clay was then filtered off and the filtrate treated according to scheme B. The coenzyme is apparently not adsorbed by the acid clay.

Fig. 8. Injection of 9 doses of coenzyme preparation "B" + vitamin B_1 concentrate. Approximately two international units of vitamin B_1 were used.

It seems from this evidence therefore that if vitamin B_1 occurred in the coenzyme it only occurred in a minute amount, and it appears doubtful whether the rise in heart rate produced was due to vitamin B_1. It is therefore unlikely that vitamin B_1 or any compound containing it participates in the oxidation of lactic acid by the systems described in this paper. Nevertheless from Peters's evidence, and from the fact that a temporary cure of the bradycardia is produced by coenzyme "B" it is possible that it is this second factor, which is missing in vitamin B_1 deficiency; but it is obviously useless to form any rigid theory until more evidence has been obtained.

Summary.

A soluble cell-free lactic dehydrogenase from heart muscle has been prepared in a less impure form than hitherto obtained. By using this enzyme it has been shown that two factors are necessary for its activation. Methods for the preparation from yeast of the first factor, which seems to be identical with Szent-

Györgyi's coenzyme, and for a mixture of the two factors have been worked out. Neither of the factors appeared to be identical with cozymase.

Using the system enzyme + lactate + mixture of two coenzymes, addition of methylene blue was found to be necessary before the system could take up oxygen.

Some evidence has been obtained which tends to show that the second coenzyme may be concerned with the removal of pyruvic acid.

The coenzymes have been obtained free from vitamin B_1 but the second factor seems to show some temporary curative effect on the bradycardia produced in vitamin B_1-deficient rats.

We wish to express our thanks to Prof. Sir F. G. Hopkins and Dr L. J. Harris for the interest they have shown in this work.

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LXXXVII. THE TRANSMISSION OF VITAMIN A FROM PARENTS TO YOUNG IN MAMMALS.

III. EFFECT OF THE FAT CONTENT OF DIET DURING PREGNANCY ON THE TRANSMISSION OF VITAMIN A TO THE FOETAL RAT.

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In an earlier paper [Dann, 1932] a preliminary investigation of the factors controlling the transmission of vitamin A from mammalian mothers to their young was described, most of the work being done upon the rat. One of the conclusions reached, which is further examined in the present communication, was that "the amount of vitamin A in the liver of the foetal rat at birth is small and subject to little variation. It cannot be increased by giving large amounts of carotene to the mother before or during pregnancy." The provitamin A, carotene, was used in those experiments because at that time no rich concentrate of vitamin A was available in the quantities required. Since the publication of the earlier paper, however, concentrates of vitamin A have become available in large quantities, and it has been thought advisable to repeat the work upon the rat using vitamin A itself in the diet. The repetition (described below) led to a conclusion similar to that reached from the earlier work, namely that the amount of vitamin A in the liver of the foetal rat remains small even when the parent doe receives large amounts of vitamin A before or during gestation.

Having established this point, it remained to be seen whether other constituents of the diet exercised any marked effect on the amount of vitamin A transmitted to the foetus during gestation when there was a liberal amount of it in the doe's diet. A factor which might *a priori* be expected to exercise some effect is the fat content of the diet, and in the present paper an investigation of the effect of the fat content of the maternal diet on the transmission of vitamin A to the foetus is described.

Experimental.

The effect of a diet rich in vitamin A on its transmission to the foetus.

For this work piebald rats from the breeding colony of this laboratory were used. The sexes were segregated at weaning and all the animals kept on the stock diet for breeding animals, consisting of bread, milk, mixed corn, green stuff and meat. At 12 weeks old, the diet was changed to one made up to resemble the carotene-rich diet used in previous experiments [Dann, 1932], which consisted of maize meal 1060, whole milk powder 400, whole grain flour 200, yeast 200, wheat germ 60, calcium phosphate 60 and salt mixture 20; for each 90 g. of this basal

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diet 10 g. of red palm oil were allowed and mixed in. In place of the red palm oil for the present experiment was substituted the same weight of a 0·5 % solution of B.D.H. vitamin A concentrate in arachis oil. When the animals were 16 weeks old, bucks and does were placed together in a single large cage, and when the does were seen to be pregnant they were removed to separate cages but kept upon the same diet. At birth of the litters both parent and young were immediately killed and the liver of the doe was removed for estimation of its vitamin A content colorimetrically with antimony trichloride. The young were examined similarly in batches of four or five instead of singly, and the vitamin A content of the carcase after removal of the liver was also determined in every case to be negative. The results of the liver examinations are set out in Table I.

Table I. *Transmission of vitamin A to foetal rats, when the parent receives much vitamin A.*

Doe	1	2	3	4	5	6
Vitamin A content of doe's liver at parturition (in B.U.)	300,000	300,000	300,000	200,000	200,000	180,000
Vitamin A content of liver of foetus at birth (in B.U.)	25	33	30	20	35	20

The effect of the fat content of the diet.

For this work also our own stock breeding animals were used. At weaning the sexes were segregated and kept in large cages on the stock diet described above.

At the age of 16 weeks, when the experimental period began, the does were divided into two groups matched in weight, and each group was put into a single large cage, together with a suitable number of bucks of the same age. One group then received a diet poor in fat and the other group a diet rich in fat, each diet containing the same liberal amount of vitamin A. In order to ensure that these conditions were strictly observed, the diets were prepared as follows. A basal diet free from fat and vitamin A was composed of 600 g. maize meal, 200 g. skim milk powder, 200 g. dried yeast and 50 g. salt mixture; weekly batches of this diet were prepared. The two experimental diets were prepared fresh daily by the addition of the requisite quantities of arachis oil and vitamin A to the basal diet. A commercial concentrate of vitamin A (obtained from The British Drug Houses, Ltd.) was taken, and 1 g. was dissolved in 19 g. of arachis oil. Each day 1·5 g. of this were weighed out and mixed into 148·5 g. of the basal diet, giving an experimental diet containing 0·05 % of the vitamin A concentrate and 1 % of fat. A further 1·5 g. of the oil was weighed out and mixed with 28·5 g. of arachis oil; the 30 g. of dilute vitamin A solution were then mixed into 120 g. of the basal diet giving an experimental diet containing 0·05 % of the vitamin A concentrate and 20 % of fat. By adopting the procedure of daily preparation of diets in this way any change in the potency of successive batches of the vitamin A concentrate is prevented from contravening the conditions stipulated above, since the two diets on any one day will always contain the same percentage of vitamin A and one will always contain twenty times as much fat as the other.

When a doe was seen to be pregnant she was at once removed to a separate cage but kept on the same diet as she had previously received. At parturition the young were removed immediately and decapitated, and the carcases were digested in aqueous KOH, from which the vitamin A was removed by extraction with ether and estimated by the antimony trichloride reaction.

Two experiments performed in this way are reported here. In the first, twelve piebald does were used; in the second, eight albino does. The results are collected in Tables II and III.

Table II. *Transmission of vitamin A to foetal piebald rats. The diet of each doe during gestation contained 0·05 % of vitamin A concentrate, but some received 1 % and others 20 % of fat.*

Doe	1	2	3	4	5	6	7	8	9	10	11	12
Fat content of doe's diet during gestation	20 %	20 %	20 %	20 %	20 %	20 %	1 %	1 %	1 %	1 %	1 %	1 %
Number of young in litter	9	8	8	10	6	9	12	8	8	10	8	8
Vitamin A store of each foetus (in B.U.)	31	25	33	35	37·5	50	25	33	25	19	25	25
Total amount of vitamin A transmitted to the whole litter (in B.U.)	279	200	264	350	225	450	300	264	200	190	200	200

Table III. *Transmission of vitamin A to foetal albino rats. The diet of each doe during gestation contained 0·05 % of vitamin A concentrate, but some received 1 % and others 20 % of fat.*

Doe	1	2	3	4	5	6	7	8
Fat content of doe's diet during gestation	20 %	20 %	20 %	20 %	1 %	1 %	1 %	1 %
Number of young in litter	9	9	12	7	6	10	10	9
Vitamin A store of each foetus (in B.U.)	11·5	37	46	16	21	30	16	9
Total amount of vitamin A transmitted to the whole litter (in B.U.)	104	243	552	112	186	300	160	81

Discussion.

The first part of the experimental work described shows that the amount of vitamin A transmitted to the foetal rat during gestation is still strictly limited when the parent animal has an enormous reserve of vitamin A in her own liver and receives a diet rich in vitamin A during gestation. The amount of vitamin A found in the foetal rats at birth was however greater in these experiments than in the author's previous experiments where the provitamin carotene was included in the diet of the parent, but it must be borne in mind that in the present experiments the does received at a moderate estimate five thousand times the necessary minimum of vitamin A in their diet.

Turning to the effect of fat in the maternal diet, it can be seen from the figures collected in Table II that the average amount of vitamin A found in the foetal piebald rats at birth was 35 B.U. when the doe received during gestation a diet containing 20 % of fat and 0·05 % of vitamin A concentrate, and 25 B.U. when the doe received a diet containing 1 % of fat and 0·05 % of vitamin A concentrate. The average figures for the total amounts of vitamin A transmitted to the whole litter during gestation were 294 B.U. and 226 B.U. for does receiving high fat and low fat diets respectively. From Table III the average figures for the albino rats are 27 B.U. and 19 B.U. transmitted to each foetus, and 253 B.U. and 182 B.U. transmitted to the whole litters, on high fat and low fat diets respectively.

From these figures it appears that an increase in the proportion of fat in the diet of a pregnant doe will in general cause an increased transmission of vitamin A to the foetus. It is plain however that with the wide differences in fat content now tested, the variation in the amount transmitted is so small (though definite) that the effect of ordinary variations in the fat content can be neglected when the diet is rich in the vitamin.

SUMMARY.

1. When the female rat receives large amounts of vitamin A (of the order of 5000 times the necessary minimum) in her diet before and during gestation, her young have only a small store of vitamin A in their livers at birth. The amount is generally between 20 and 35 B.U. of the vitamin. In the body apart from the liver no vitamin A can be detected.

2. The amount of vitamin A transmitted to the foetal rat during gestation when the parent doe receives large amounts in her diet is affected by the fat content of the diet and is increased as the fat content increases. This increase in transmission is small and for ordinary ranges of variation of fat content is probably negligible.

I wish to express my thanks to Dr T. Moore for his interest in this work and for many suggestions towards its furtherance.

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LXXXVIII. ASCORBIC ACID IN THE EYE-LENS AND AQUEOUS HUMOUR OF THE OX.

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(*Received March 2nd, 1934.*)

In an earlier communication [Birch and Dann, 1933] on the estimation and distribution of ascorbic acid and glutathione in animal tissues we drew attention to the distribution of ascorbic acid in organs other than the suprarenal cortex, as detected by the indophenol reagent. The presence in a number of tissues of reducing substances other than glutathione had become evident from the work of Mason [1930] and of Bierich and Rosenbohm [1933], but no evidence of the identity of such substances had been obtained. By applying the indophenol titration for ascorbic acid to tissue extracts we were able to demonstrate the presence of the acid in a number of organs and to give figures for the amounts present. At the same time it was pointed out that the indophenol titration may not be specific for ascorbic acid in tissue extracts, so that other tests are necessary to confirm the results obtained by the titration method.

The silver nitrate staining test was applied to a number of the tissues which had been found by means of the indophenol titration to contain ascorbic acid, with results which were not always in harmony with those obtained by the latter method. Some tissues found by the titration method to contain ascorbic acid gave little or no staining with silver nitrate, and led us to the conclusion already formulated by Harris and Ray [1933, 1] that a positive result with this test indicates the presence of ascorbic acid in a tissue but a negative result does not necessarily mean that ascorbic acid is absent.

In order to examine the reliability of results obtained by the titration method it was decided to carry out biological assays of the ascorbic acid found by the titration method to occur in unexpected sites. The tissues chosen for examination were the eye-lens and aqueous humour of the ox.

Experimental.

For the first attempt to assay biologically the ascorbic acid in the lens and the aqueous humour of the ox's eye the recovery test of Harris *et al.* [1932] was employed. Young guinea-pigs fed on a full diet of natural foodstuffs on attaining the weight of 300 g. were placed on a vitamin C-free diet consisting of bran 80 parts, ground oats 720, egg-yolk 40, salts 84, cod-liver oil 1 %, and were weighed daily thereafter. As soon as any animal showed a loss in weight on two successive days, it was given its first dose of the material under test and then dosed daily. The weight curves of the animals are shown in Fig. 1.

Since no simple method of extracting the ascorbic acid from the lens could be devised, the method of preparing the lens for dosing was to remove the lenses

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from a number of ox eyes which had been kept in cold storage after the slaughtering and to estimate by the indophenol micro-titration the amount of ascorbic acid in a sample of the lenses. Weighed amounts of the lenses calculated to contain (from the titration results) either 1 mg. or 3 mg. of ascorbic acid were then mashed up in a little water until the suspension could be taken up in a pipette and given to the guinea-pig. For doses of the aqueous humour, the humour was extracted from a number of eyes and pooled; an ascorbic acid

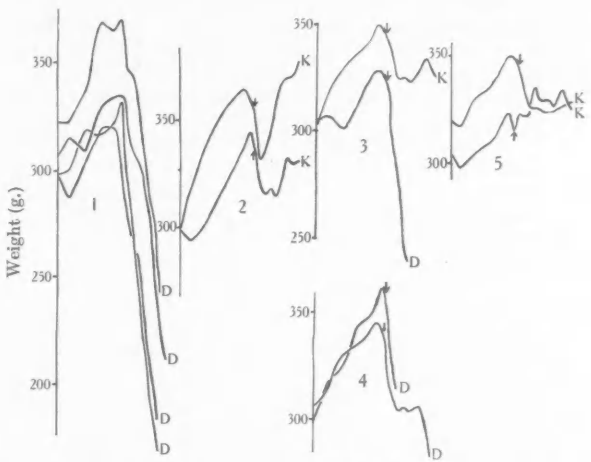

Fig. 1. Weight curves of guinea-pigs used in recovery test. The daily doses of the different groups were as follows: (1) No addition to basal diet; (2) 3·0 ml. of orange juice; (3) Eye-lens calculated from indophenol titration to contain 1 mg. ascorbic acid; (4) Eye-lens calculated to contain 3 mg. ascorbic acid; (5) Aqueous humour calculated to contain 2 mg. of ascorbic acid. Dosing commenced as indicated by the arrows. D signifies died; K signifies killed.

determination was then made on a portion of the mixed humour. The amount of humour calculated to contain 2 mg. ascorbic acid was then given to the guinea-pig from a pipette. The ascorbic acid estimation and preparation of doses were carried out each day, and a fresh supply of eyes was obtained as often as possible (generally every day).

From the weight curves in Fig. 1 it can be seen that the test was not successful. Three of the four animals receiving a supplement of eye-lens died before the first of the negative control animals, the average time between commencement of the experimental period and death being $4\frac{1}{4}$ days for the animals receiving eye-lens and $6\frac{1}{2}$ days for the negative controls. Thus no evidence of the presence of ascorbic acid in the lens is afforded by this test, which however suggests not so much the absence of ascorbic acid from the lens as the presence of some other substance exerting a positive toxic effect. The animals receiving aqueous humour lived through the experimental period of 9 days but did not show an increase of weight, as did the positive controls receiving 3 ml. of orange juice daily. The curves indicate that the dose of aqueous humour contained about 1·2 mg. ascorbic acid, or about two-thirds of the amount found by the titration. If the aqueous humour exerts in slighter degree a similar toxic action to the lens, then

the amount of ascorbic acid present would be greater than is indicated by the curves.

On account of the failure of this test with the lens a second test was arranged with alterations considered likely to diminish the effect of the toxic action of the lens. Instead of the curative test, the tooth structure method of Höjer as modified by Key and Elphick [1931] was used, as it has the advantage that at the beginning of the dosing period the guinea-pigs have not been entirely depleted of their vitamin C and therefore are likely to be more resistant to the toxic action of the dosing material. For convenience of dosing a large batch of ox eyes was obtained, and the lenses were removed, dried in a vacuum-desiccator and powdered. The dried powdered lenses were well ground and mixed to give a uniform material, which by titration against the indophenol was found to contain 0·9 mg. ascorbic acid per g. The indophenol reagent was standardised against pure ascorbic acid. The dose given to each animal daily was 3 g. of the powdered eye-lens, containing according to the titration 2·7 mg. of ascorbic acid. Four animals were used, together with three negative controls, four animals receiving 3·5 ml. orange juice daily and three animals receiving 2 ml. orange juice daily. After 14 days all the animals were killed and their lower jaws dissected out for examination of the teeth. During the dosing period one animal receiving the eye-lens had gained a little in weight, one had remained steady and the other two had fallen considerably. The results of the histological examination of the teeth are set out in Table I.

Table I.

Guinea-pig no.	Daily dose	Degree of protection from scurvy*	
1	3 g. dried eye-lens	2·6	
2	,,	2·2	Mean 2·5
3	,,	2	
4	,,	3·3	
5	3·5 ml. orange juice	2·3	
6	,,	3·5	Mean 3·2
7	,,	3	
8	,,	3·8	
9	2 ml. orange juice	1·5	
10	,,	1	Mean 1·4
11	,,	1·8	
12	None	0	
13	,,	0·3	Mean 0·5
14	,,	1·2	

* Each figure in this column is the mean of three, allotted by three independent observers.

From the figures given it appears that ascorbic acid is undoubtedly present in the lens of the ox eye. The doses of orange juice given to the positive control animals were chosen to contain 2·1 mg. and 1·2 mg. of ascorbic acid in 3·5 ml. and 2·0 ml. respectively. Since this work was undertaken, however, it has been demonstrated by Harris and Ray [1933, 2] that the ascorbic acid content of the juice of different oranges varies a good deal more than was supposed. Hence it is difficult to give a quantitative figure for ascorbic acid content of a test dose used in a biological test unless the positive controls have been dosed with pure ascorbic acid. On the assumption that the orange juice used in our experiments was near to the average in ascorbic acid content (*i.e.* 0·6 mg. per ml. of juice) then the results set out in Table I indicate that the eye-lens contains ascorbic acid to the extent of about two-thirds of the amount suggested by indophenol titration.

SUMMARY.

The eye-lens and aqueous humour of the ox have been tested biologically by the tooth protection method and the recovery method respectively, and the presence of ascorbic acid found by the indophenol titration method has been confirmed.

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LXXXIX. FURTHER STUDIES ON THE LACTIC ACID, SUGAR AND INORGANIC PHOSPHORUS OF THE BLOOD OF RUMINANTS, (*a*) FOLLOWING ADRENALECTOMY AND (*b*) AFTER INTRAVENOUS INJECTION OF INSULIN.

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In a previous paper [Allcroft and Strand, 1933] we have shown that a marked increase occurs in the percentages of lactic acid and sugar in the blood of sheep after muscular exercise and/or excitement, and it seemed important to determine whether these changes would also occur after adrenalectomy. While there are numerous references to the effect of adrenalectomy in dogs and small laboratory animals, we can find only one reference to similar work in the ruminant. Moore and Purinton [1901], working on two goats and two kids, found that the operation was fatal in three out of the four cases.

Briggs *et al.* [1923–24] state that after insulin injections in normal animals, there is a decrease in glucose, phosphorus and potassium in the blood with an increase in blood-lactic acid. Best and Ridout [1925] state that the lactic acid content of the blood of dogs does not significantly increase during insulin hypoglycaemia. Cori [1925] reports that insulin hypoglycaemia was attended by no definite changes in the lactic acid of either fasting rabbits or cats: later, however [1931; 1932], this worker reports an increase in lactic acid and a decrease in phosphorus after injection of insulin in normal rabbits, but after adrenalectomy these changes were not observed. Katayama and Killian [1926–27] also conclude that insulin produces an increase in the lactic acid of the blood of rabbits and human beings and note that in the rabbit the changes in concentration of lactic acid are very slight in comparison with the drop in blood-sugar. These rather contradictory reports led us to attempt to study the changes in lactic acid, sugar and inorganic phosphorus of the blood of sheep after injection of insulin. Though it was not possible to complete the study it is thought advisable to report the observations which were made.

Technique.

The sheep were brought in from grazing and put in metabolic cages for 18–24 hours before operation. Food was withheld during this period but water was available. The animals were anaesthetised by ether and the wool was cut short, but not shaved, over the skin of the abdomen between the costal margin and the umbilicus. An oblique incision (about 8 in. long) was then made in the loin stretching from the lower border of the 12th rib on the right side

towards the umbilicus. By retraction of the viscera, the adrenal gland was readily exposed and the small vein crossing in front of it from the parietes to the inferior vena cava was doubly ligated. Narrow tapes were then placed with care under the vena cava above and below the site of the gland. By gently pulling on these it was possible to control the bleeding during the removal of the gland, which was the next step. After removal, any obvious small veins or arteries were tied and the longitudinal slit which is unavoidably made in the vena cava, as a result of the excision of the gland (on account of the short length of the adrenal vein) stitched by fine linen thread on fine needles, and a small piece of wet cotton wool placed over the suture line to prevent leakage. The abdominal wound was then closed and the animal allowed to recover from this first operation. The removal of the left adrenal gland followed in 8–20 days. In this case the length of the adrenal vein was sufficient to permit of the removal of the gland without damaging the vena cava. The animals recovered well from both operations, without any suppuration of the wounds.

In a few cases cortico-adrenal extract was injected after bilateral adrenalectomy to maintain the animal in good condition. The extract was made by Grollman and Firor's method [1933] and assayed by intraperitoneal injection into month-old adrenalectomised rats. If the extract were potent the rats gained 1–4 g. in weight daily and appeared to be in a normal condition. Occasional batches of extract were not potent, and after injection of these the rats lost weight daily showing typical adrenal insufficiency symptoms such as loss of appetite, muscular weakness, general apathy and often convulsions just before death, which resulted in 2–4 days after the operation.

Although the extracts were as a rule potent as judged by their effect on growing rats, it is not certain, owing to the limited number of experiments, whether their injection into adrenalectomised sheep really prolonged life (see Table I). We gained the impression however that the injected animals were in better condition than the controls.

Owing to the short survival period of bilaterally adrenalectomised sheep it was found necessary to carry out the excitement experiments as soon as possible and the second day after the final operation was chosen since by that time the blood-sugar had assumed a low level. Blood samples were taken from most of the animals before unilateral adrenalectomy, in the period between unilateral and complete adrenalectomy, and after the final operation. The technique and times of sampling were essentially the same as in the earlier work except that sampling was continued for only 2 hours following the excitement, since this period covered the major changes which we had noted.

In the insulin injection experiments, insulin was injected into the left jugular vein and samples of blood were taken from the right jugular vein, before injection and at intervals of 15, 30, 45, 60, 90, 120, 180, 240 and 300 minutes afterwards. The amount of insulin injected per kg. body weight is given in each graph. In the case of the one adrenalectomised sheep to which insulin was administered it was not possible to obtain the full series of samples owing to convulsions, which occurred 50 minutes after the insulin injection.

Lactic acid was estimated by the method of Friedemann *et al.* [1927], blood-sugar by the Hagedorn and Jensen [1920] method and inorganic phosphorus by Briggs method [1922]. For all estimations the blood-proteins were precipitated within 1–2 minutes after the blood sample was drawn.

The results of parts (*a*) and (*b*) are given and discussed separately.

(*a*) The following table (Table I) gives the results of adrenalectomy on the blood constituents.

Table I.

Results expressed in mg./100 ml. whole blood.

Sheep No.	Before unilateral adrenalectomy: Lactic acid	Blood-sugar	Inorg. P	After unilateral adrenalectomy: Lactic acid	Blood-sugar	Inorg. P	After complete adrenalectomy: Lactic acid	Blood-sugar	Inorg. P	Survival period in (hours)	Remarks
167	15·23	61·80	—	10·40	57·40	—	5·80	45·96	8·44	52	Left gland removed 8 days after right. In good condition first day after complete adrenalectomy. Insufficiency symptoms set in about 9 a.m. second day. No C-A extract injected
	15·57	60·04	—	10·12	61·80	7·76	7·00	40·68	8·64		
	—	—	—	7·72	61·80	7·76	6·05	35·40	9·64		
	—	—	—	—	—	—	8·95	40·68	9·64		
125	—	—	—	22·05	79·40	—	8·45	35·40	—	58	Left gland removed 10 days after first operation. In good condition first day after complete adrenalectomy. On second day insufficiency symptoms were marked. No C-A extract injected
	—	—	—	19·50	81·16	—	6·26	35·40	—		
	—	—	—	20·20	—	—	8·45	37·16	10·72		
	—	—	—	—	—	—	8·45	34·52	11·64		
1	17·45	55·64	3·94	—	—	—	—	—	—	—	Left gland removed 7 days after first operation. Died a few hours after second operation due to haemorrhage from inferior vena cava
	15·50	52·12	4·21	—	—	—	—	—	—		
	13·30	52·00	4·47	—	—	—	—	—	—		
2	47·70	68·84	6·08	—	—	—	(1) 11·10	41·56	10·64	34	Left gland removed 8 days after right. In good condition day after complete adrenalectomy. Two blood samples taken 9.30 and 10 a.m. (Nos. 1 and 2). Then 10 ml. of C-A extract injected intraperitoneally. Two more samples (3 and 4) taken 15 and 30 minutes after injection. No insufficiency symptoms apparent but sheep died a few hours after injection of toxic extract No. 5*
	17·70	73·24	5·42	—	—	—	(2) 13·75	44·20	9·86		
	27·90	74·12	5·42	—	—	—	(3) 13·75	65·32	10·80		
	—	—	—	—	—	—	(4) 14·50	66·20	11·16		
3	15·00	71·48	6·03	12·30	64·44	4·42	7·60	47·72	7·30	60	Left gland removed 18 days after first operation. In good condition next day and following day during which it received 30 ml. extract in 5 ml. injections. Was in good condition 6.30 p.m. on second day but collapsed suddenly and died 11 p.m. same night
	13·05	74·12	5·32	19·00	60·04	5·09	6·40	38·92	8·14		
	14·25	73·24	5·44	20·50	66·20	5·17	7·25	43·32	7·89		
4	18·30	61·80	5·53	11·80	62·68	2·79	—	—	—	—	Left gland removed 16 days after first operation. Died the morning following the second operation due to tying of the vena cava
	11·10	62·68	4·98	9·70	65·32	2·79	—	—	—		
	12·56	62·68	4·66	10·40	68·84	2·56	—	—	—		
5	24·00	70·60	5·65	—	—	—	(1) 11·40	54·76	7·94	52	Left gland removed 6 days after right. 5 ml. C-A extract (No. 11) injected at hourly intervals during day following complete adrenalectomy. Animal in very good condition. Samples 1 and 2 taken day after second operation and samples 3 and 4 the next morning. After No. 4 sample was taken insulin (0·75 units/kg. body weight) was injected. 50 minutes afterwards animal had convulsions. C-A extract and large quantities of glucose solution were injected but sheep did not recover and died 7 hours after insulin injection
	26·70	69·72	5·60	—	—	—	(2) 11·60	54·76	7·68		
	26·00	70·60	5·60	—	—	—	(3) 12·15	62·68	12·24		
	—	—	—	—	—	—	(4) 10·40	55·64	13·00		

* This extract was found to be highly toxic by injecting 0·5 ml. of it intraperitoneally into mice.

The effects of excitement are shown in the curves of Figs. 1–4.

Sheep Nos. 167 and 125 (Figs. 1 and 2) were subjected to a barking dog but showed singular lack of interest in the proceedings. After the 120 minute sample had been taken one of the sheep (No. 125) was exercised for 5 minutes and then samples taken immediately and 15 minutes after. The dog barking caused no significant alteration in the level of any of the three constituents studied in

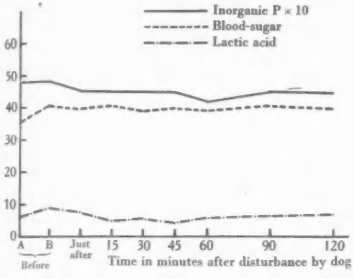

Fig. 1.

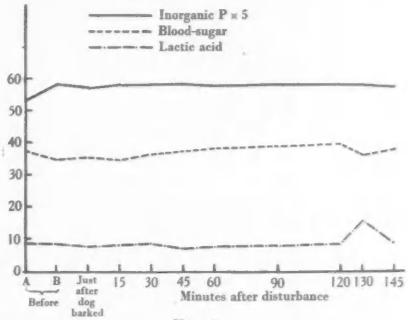

Fig. 2.

Fig. 1. Effect of excitement (dog barking) on sheep No. 167 bilaterally adrenalectomised. Ordinate: mg. per 100 ml. whole blood.

Fig. 2. Effect of excitement (dog barking) on sheep No. 125 bilaterally adrenalectomised. 10 minutes' exercise between 120 and 130 minutes. Ordinate: mg. per 100 ml. whole blood.

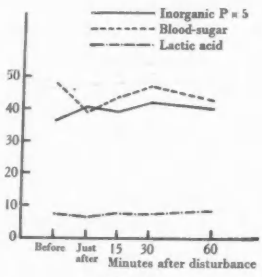

Fig. 3.

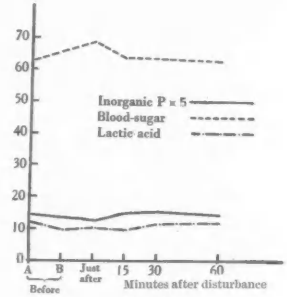

Fig. 4.

Fig. 3. Effect of excitement (by explosion of cracker) on sheep No. 3 bilaterally adrenalectomised. Ordinate: mg. per 100 ml. whole blood.

Fig. 4. Effect of excitement (by explosion of cracker) on sheep No. 4 unilaterally adrenalectomised. Ordinate: mg. per 100 ml. whole blood.

either animal, and the changes in lactic acid and sugar which occurred in sheep No. 125 after exercise are so small as to be of doubtful significance (Fig. 2).

Sheep Nos. 3 and 4, the former bilaterally, and the latter unilaterally adrenalectomised, were subjected to the explosion of a medium-sized firework placed about 4 ft. from them. Sheep No. 3 (Fig. 3) showed no alteration in lactic acid or inorganic P but the curve for sugar showed a lower level after the explosion, returning to the pre-experimental level within 30 minutes. In sheep

No. 4 the only change was a slight elevation of the blood-sugar immediately after the explosion (Fig. 4). This increase in blood-sugar and the normal low level of the inorganic P is, in our opinion, due to the fact that the animal is not completely adrenalectomised.

Discussion.

(*a*) It can be seen from the above results that the blood-lactic acid was lower after complete adrenalectomy than before or after unilateral adrenalectomy. This is contrary to the conclusions of Britton and Silvette [1932] with cats, but in agreement with those of Hastings and Compere [1930–31] with dogs. The blood-sugar level was also considerably lowered after complete adrenalectomy. Injection of C-A extract definitely raised the blood-sugar in one sheep, No. 2, and possibly diminished the fall in another, No. 5 [*cf.* Britton and Silvette, 1932; Wyman and Walker, 1929].

The inorganic P was considerably raised after complete adrenalectomy. It was observed too in every case after the second gland was removed that there was a marked increase in the blood concentration so that it was often difficult to get a series of blood samples. Just before death the usual symptoms of adrenal insufficiency were present, *viz.* refusal of food, muscular weakness, prostration, decrease in body temperature and finally convulsions. These observations, increase in inorganic P, anhydraemia and insufficiency symptoms, agree with those made by Britton and Silvette [1932], Britton [1932] and Lucas [1926].

Banting and Gairns [1926] state that the lowering of the blood-sugar does not depend on the absence of the adrenals but is the result of the shock of the operation. If this were so one would expect a gradual rise in the blood-sugar as the animal recovered from the operation, but in no instance was this observed. None of the animals survived for a longer period than 60 hours after complete adrenalectomy. In the cases where cortico-adrenal extract was injected (except in sheep No. 5) the short survival period was probably due to the fact that the extract was not sufficiently potent to prolong the life of the animal. On the same extract however we were able to keep completely adrenalectomised month-old and adult rats indefinitely. It is extremely probable that sheep No. 5 died of insulin shock since it has been shown that adrenalectomy renders other animals hypersensitive to insulin [Ellsworth and Weinstein, 1933; Tada, 1931; Lewis and Magenta, 1925; Houssay and Lewis, 1923]. Moore and Purinton [1901] record survival periods for two bilaterally adrenalectomised kids of 49 and 60 hours, while one goat survived 8 days and in another the results were not fatal.

Effect of excitement. It is evident that in completely adrenalectomised sheep no significant alteration occurs in the levels of lactic acid, sugar or inorganic P of the blood after the same type of emotional stimuli which were found to cause marked rises in normal sheep [Allcroft and Strand, 1933]. This confirms the views of Lumley and Nice [1930], who showed that after excitation induced by shocks sufficient to cause struggling in adrenalectomised rats, no increase in the blood-sugar occurred, while in normal rats under similar conditions increases up to 86 % were observed. Britton [1933] also reports that none of the measures which normally produce hyperglycaemia in cats, *e.g.* adrenaline, anaesthesia or excitement, has any significant influence on the blood-sugar level of adrenalectomised animals.

(*b*) Figs. 5–8 show graphically the changes which occurred after insulin injection in the four unoperated sheep. Figs. 5 and 7 show results obtained on the same sheep with an interval of 16 days between the experiments.

In Table II are given the results of the few blood samples which were secured from the completely adrenalectomised sheep, and also in chronological sequence the therapeutic treatment administered during the convulsive stages. Though

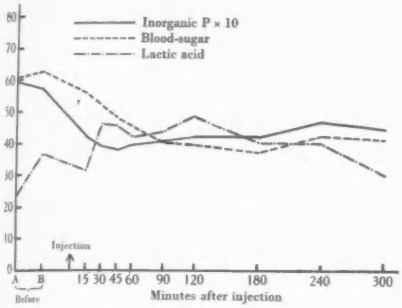

Fig. 5.

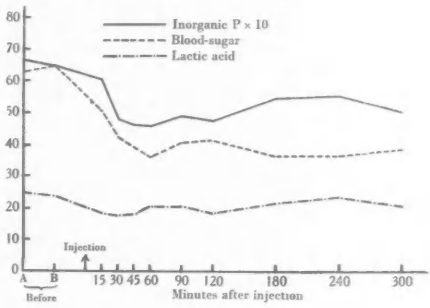

Fig. 6.

Fig. 5. Effect of intravenous injection of insulin (2 units per kg.) on normal sheep No. 9. 25. ix. 33. Ordinate: mg. per 100 ml. whole blood.

Fig. 6. Effect of intravenous injection of insulin (2 units per kg.) on normal sheep No. 13. 25. ix. 33. Ordinate: mg. per 100 ml. whole blood.

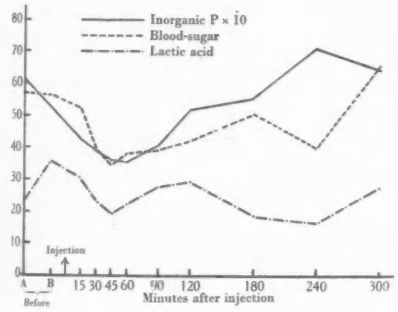

Fig. 7.

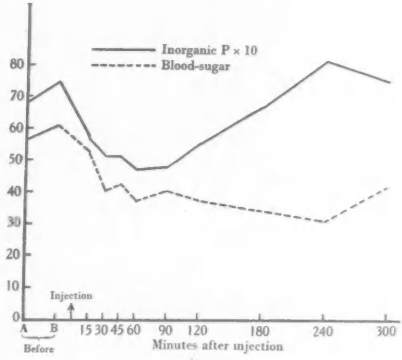

Fig. 8.

Fig. 7. Effect of intravenous injection of insulin (2 units per kg.) on normal sheep No. 9. 11. x. 33. Ordinate: mg. per 100 ml. whole blood.

Fig. 8. Effect of intravenous injection of insulin (3 units per kg.) on normal sheep No. 10. 11. x. 33. Ordinate: mg. per 100 ml. whole blood.

the animal had been maintained in good condition with cortico-adrenal extract up to the commencement of the experiment the subsequent injections of glucose and cortico-adrenal extract were unable to prevent death 7 hours after injection of insulin.

It will be seen that in only one case (Fig. 5, sheep No. 9) did the blood-lactic acid show any definite increase after intravenous injection of insulin. The samples obtained at 45 and 300 minutes after injection of insulin into the adrenalectomised sheep (see Table II) gave high values for lactic acid associated

Table II. *Effect of insulin injection on a completely adrenalectomised sheep.*

27·2 kg. weight (0·75 unit insulin per kg.) 20·4 units injected.
Results expressed in mg./100 ml. whole blood.

	Lactic acid	Blood-sugar	Inorganic P	Remarks
Before injection	12·15	62·68	12·24	—
	10·40	55·64	13·00	Injection of 0·75 unit insulin per kg. body weight
15 mins. after	11·60	45·96	12·16	—
30 ,,	10·05	40·68	11·54	—
45 ,,	23·75	36·28	12·50	—
50 ,,	—	—	—	Convulsions began. 11 ml. glucose intravenously
70 ,,	—	—	—	11 ml. glucose intravenously
83 ,,	—	—	—	11 ml. glucose intravenously
95 ,,	—	—	—	11 ml. glucose intravenously + 0·5 ml. adrenaline subcutaneously
210 ,,	—	—	—	60 ml. glucose subcutaneously
300 ,,	20·10	57·40	10·98	—

with the hypoglycaemic convulsions. The changes in the blood-sugar correspond to those reported in the literature, although the degree of hypoglycaemia produced was not the same in all cases, and the low level seemed more prolonged in the ruminant than in simple-stomached animals. The fall in inorganic P agrees with the work of Cori and Cori [1932] on rabbits and of Eadie *et al.* [1925] on dogs and rabbits. The former observed that the level was lowest 45 minutes after injection and gradually returned to the original level. Eadie *et al.* [1925] state that the blood-phosphate fall is simultaneous with that of blood-sugar, but the rise to normal occurs before that of sugar and in some cases may continue above the normal level. In our own work the lowest levels lie between 45 and 60 minutes after injection and in two cases (Figs. 7 and 8) there was a rise to above the normal level. It is possible that this return to a higher level was delayed in Figs. 5 and 6 and might have occurred after the last samples were taken. Ellsworth and Weinstein [1933] observed similar phenomena in dogs whether normal, or completely adrenalectomised and maintained on cortical extract: one adrenalectomised sheep however showed only a very slight fall in inorganic P.

It is obvious that adrenalectomy increases the sensitivity of the sheep to insulin just as it does that of other animals. At the same time it is evident that the normal ruminant can tolerate much larger doses of insulin without occurrence of convulsions than can simple-stomached animals. Peterson *et al.* [1931] report that they lowered the blood-sugar in cows to values as low as 17 mg. per 100 ml. by insulin injections and yet failed to induce convulsions.

Summary.

1. A description is given of the surgical technique used for adrenalectomy operations on sheep.

2. After bilateral adrenalectomy in sheep the blood-sugar and lactic acid levels were definitely lowered while that of inorganic P was raised.

3. No change occurred in blood-sugar and lactic acid of adrenalectomised sheep when they were subjected to such types of excitement and muscular exercise as were known to raise them in normal sheep.

4. There was no definite change in the blood-lactic acid of normal sheep after insulin injections.

5. The hypoglycaemia produced by insulin injection in normal ruminants was similar to that in other animals except that no sign of convulsions was observed even after large doses of insulin.

6. The changes in blood-inorganic P after insulin corresponded to those found in other animals.

7. Bilateral adrenalectomy increases the sensitivity of the ruminant to insulin.

We wish to express our thanks to Prof. J. J. R. Macleod for his advice and interest in this work, to Dr R. A. Cleghorn and Dr H. E. Magee for assistance in part of it.

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XC. THE ULTRAFILTRATION OF PROTEINS THROUGH GRADED COLLODION MEMBRANES.

I. THE SERUM-PROTEINS.

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Many qualitative investigations showing the influence of various physical factors upon the process of ultrafiltration are contained in the literature. Elford [1933] has discussed these effects in a more quantitative manner, based on experimental results with graded collodion membranes [Elford, 1931]. The latter afford a unique advantage in that they provide a wide selection of grades and can be accurately calibrated in terms of average pore diameter; and groups of membranes known to be of the same grade may be used in comparative filtrations with different systems. The present study considers the influence upon the ultrafiltration of proteins of the nature of the solvent medium, its hydrogen ion concentration, the concentration of protein and filtration pressure. It establishes a firmer basis for the standard conditions which have been adopted as being most favourable for ultrafiltration studies on viruses and bacteriophages in this Laboratory (for a partial bibliography see Burnet and Andrewes [1933]). It confirms the values previously presented for the empirical correction factor used in estimating the particle size of a virus or phage from the pore diameter of the limiting membrane which, under those optimum conditions, just completely retains it.

Materials.

The proteins used were highly purified samples of horse serum-albumin and pseudoglobulin, kindly presented to us by Dr H. W. Dudley, to whom we acknowledge our indebtedness. The pseudoglobulin was prepared by precipitation from normal horse serum with half-saturated ammonium sulphate, and subsequent dissolution in water and precipitation with ammonium sulphate three times. The final precipitate was dissolved in water and dialysed under slight pressure. This served to precipitate any remaining euglobulin, which was sedimented by centrifuging. The clear centrifugate, containing the pseudoglobulin, was concentrated *in vacuo* over sulphuric acid to obtain the solid protein. The albumin, contained in the first filtrate from the globulin precipitation, was precipitated three times by the method of Hopkins and Pinkus [1898], the requisite amount of acetic acid for producing a crystalline precipitate being determined in each case by trial. The final precipitate was redissolved in water and dialysed and concentrated *in vacuo* as above.

When the albumin was dissolved in water, or the pseudoglobulin in 0·05 % sodium chloride solution, practically no insoluble residue remained. Protein-free ultrafiltrates of solutions of both proteins in distilled water contained no chloride

detectable by the silver nitrate test and only a trace of sulphate as detected by barium chloride. The ash contents of the solid samples were: albumin, 0·8 %; pseudoglobulin, 0·4 %.

Procedure.

Exclusive use was made of the graded collodion ("gradocol") membranes, prepared and calibrated by the methods of Elford [1931] and Elford and Ferry [1934]. Nickel-plated ultrafilters of the type described by Barnard and Elford [1931] were slightly attacked by the more acidic protein solutions, and accordingly silver-plated models were employed. The components of the apparatus were sterilised separately and assembled under sterile conditions. Filtration was carried out under positive pressure of from 1 to 3 atmospheres of nitrogen. In comparative experiments, six filtrations could be run simultaneously from a pressure distributor attached to one nitrogen cylinder.

In a typical experiment, a solution of protein was made up to the desired concentration in a given solvent medium, and the p_H was adjusted, if necessary, by addition of small quantities of 0·1 *N* or *N* hydrochloric acid or sodium hydroxide. The solution was then filtered through a membrane of grade between 0·5μ and 1·0μ to sterilise it. Filtration through such a membrane affords a very convenient method for removing bacteria and gross aggregates with minimum loss of protein by adsorption. This sterile stock filtrate could be kept for months at 4° without any appreciable change in appearance, p_H (unless originally alkaline, when absorption of CO_2 from the atmosphere soon shifted the reaction), or filtrability. In no case was an antiseptic preservative necessary. When a comparison of different media was required, the stock was made up to twice the final concentration desired, and aliquot parts were diluted with the different media.

Different samples, usually 10 ml. of stock solution, were filtered through various membranes, all of comparable thicknesses and of grades ranging from that which let the protein through in nearly undiminished concentration to that which yielded filtrates giving a negative test with sulphosalicylic acid. For each individual filtration, the filtrate was collected in successive ml. samples and analysed with a Zeiss dipping refractometer. The concentrated residue remaining over the membrane was also analysed. The refractometric analyses were roughly checked by precipitation with sulphosalicylic acid, which was sensitive to a concentration of about 0·005 % protein. The relative concentration, *u*, of the

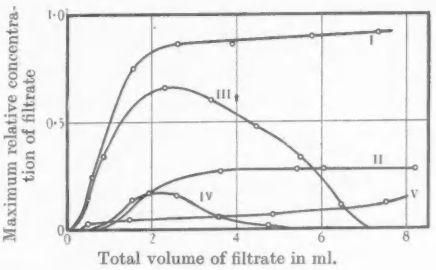

Fig. 1. Typical filtration curves.

filtrate, in terms of the concentration of protein in the stock solution (using the refractive index of the protein-free ultrafiltrate as a zero-point) could be expressed with an accuracy of about 2 %. It was plotted against the total volume filtered to give the "filtration curve" (Fig. 1).

The highest point on this curve, u_{max}, was characteristic of the solution and the membrane and was reproducible for comparable conditions to 5–10 %. The value of u_{max} is largely determined, in curves of type I, by the amount of adsorption of protein by the membrane; in curves of type II, by a process not fully explained, perhaps a true sieving mechanism; in curves of types III and IV, by the stage at which the narrowing and blocking of pores begins. These processes are discussed more fully below. In curves of type V, u is still rising at the end of the experiment, and u_{max} is significant only in comparing different filtrations which have all been carried to the same point.

Values of u_{max} for different membranes were plotted against the logarithm of membrane average pore diameter, log j, to give the "end-point curve" (Fig. 2). The end-point, j_e, for the disperse phase in a given system is the A.P.D.[1] of the membrane which just fully retains it.

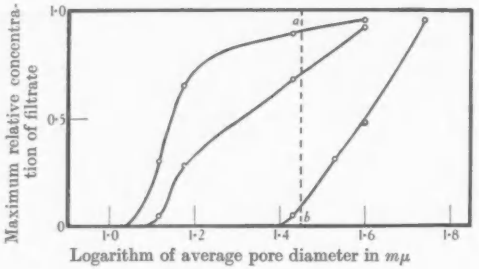

Fig. 2. Typical end-point curves.

The slopes of the end-point curves vary for different systems, as shown in Fig. 2. Hence the "filtrability" of the disperse phase in a given system cannot well be characterised by the end-point alone; it should be represented by the whole curve. We have selected three points on the curve for a convenient measure of filtrability: the values of average pore diameter, $j_{0·5}$, $j_{0·2}$ and j_e, which correspond with the maximum relative concentrations of filtrates 0·50, 0·20 and 0 respectively. The higher these values are, the less "filtrable" is the system.

The filtrabilities of different systems may be compared among themselves, however, by the values of u_{max} for one given grade of membrane, represented by the intersections of the end-point curves with the vertical line drawn through the appropriate value of log j, as shown in Fig. 2 by ab.

General effect of the nature of the solvent medium on filtrability.

The effect of capillary-active substances in decreasing the adsorption zone of the filtration curve and favouring filtration generally has been discussed [Elford, 1933]. The suspending medium uniformly used for filtration studies on viruses and bacteriophages in this Laboratory has been the Hartley's broth which is employed in bacteriological work. The stock broth is prepared from horse-flesh by extraction and digestion with trypsin [Hartley, 1922]. Its effect in facilitating filtration may be due to (1) peptisation of the particles of the disperse phase by adsorption of large residues of broken-down proteins; (2) lowering of surface tension of the solution; (3) peptisation and electrokinetic effects due to the presence of neutral salts.

[1] The abbreviation "A.P.D." is conveniently used for *average pore diameter*.

The stock broth often contains proteoses; addition of sulphosalicylic acid produces a precipitate which, in contrast with that given by the large serum-proteins, may be redissolved by boiling. Before investigating the influence of broth upon the filtrabilities of the serum-proteins, an effort was made to obtain a proteose-free broth. The standard method of preparation was modified by prolonging the digestion with 5 % Cole and Onslow's pancreatic extract for 2 weeks at 37°. Chloroform was added as a preservative. The resulting "extra-digested" broth gave a negative test with sulphosalicylic acid. Its surface tension at 21° was about 6 % lower than that of water, compared with a 25 % to 30 % lowering in the case of the stock broth. Its frothing power was greatly diminished.

Experimental.

Comparative experiments were made to show the effects of various media on filtrability. Solutions of serum-albumin, concentration 0·5 %, were made up in water, 1 % sodium chloride, and 1 : 1 dilutions in water of extra-digested broth, two different preparations of stock broth, and ox-heart broth (*i.e.* Hartley's broth prepared from ox-heart instead of horse-flesh). Each system, adjusted to approximately the same p_H, was filtered through a membrane of the same A.P.D. A similar experiment was performed with the pseudoglobulin, using 0·05 % sodium chloride solution instead of water. The results are shown in Table I.

Table I. *Filtration of serum-proteins in various solvent media.*

Concentration of protein 0·5 %.

Medium	Albumin. Membrane A.P.D. 14·5$m\mu$		Pseudoglobulin. Membrane A.P.D. 59$m\mu$	
	p_H	$u_{max.}$	p_H	$u_{max.}$
Water	7·1	0·14	—	—
0·05 % NaCl	—	—	7·1	0·14
1·0 % NaCl	6·9	0·34	7·3	0·61
Extra-digested broth	7·1	0·36	7·3	0·57
Stock broth, preparation 26–9	7·5	0·46	7·4	0·81
Stock broth, preparation 24–10	7·4	0·33	7·3	0·74
Ox-heart broth	7·4	0·46	7·4	0·73

Results. The data demonstrate the superiority of Hartley's broth as a solvent medium in the filtration of both proteins. Stock and ox-heart broths favour the filtration to about the same extent and are definitely more effective than extra-digested broth, which in turn has about the same effect as the 1 % sodium chloride solution. The superiority of the stock and ox-heart broths over the extra-digested preparation is probably due to the presence in the former of a capillary-active component, which becomes destroyed by prolonged digestion. It is also to be noted that in every instance the improvement in filtration is more marked for pseudoglobulin than for albumin. The mechanism of these effects will be discussed below.

Effect of p_H on filtrability.

Serum-albumin.

Experimental. The complete filtration and end-point curves were determined for filtrations of solutions of serum-albumin, concentration 0·4 % to 0·5 % in distilled water, at different values of p_H ranging from 3·0 to 8·8. The characterising

A.P.D. values, $j_{0\cdot5}$ and $j_{0\cdot2}$, were obtained by interpolation, and j_e by extrapolation on each curve. These three quantities are plotted against the p_H in Fig. 3.

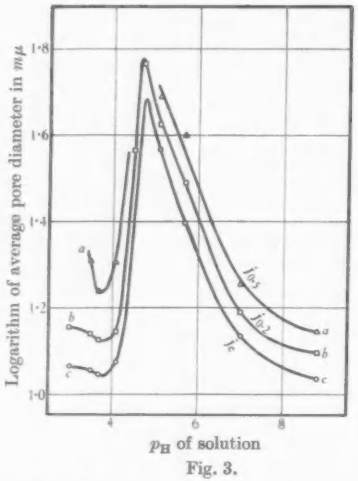

Fig. 3.

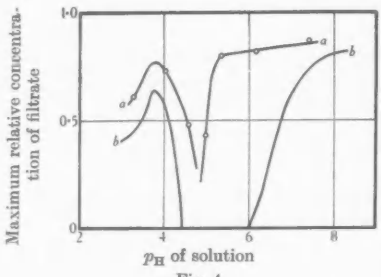

Fig. 4.

Fig. 3. p_H-filtrability curves of serum-albumin dissolved in water. *a*. $u_{max}=0\cdot50$. *b*. $u_{max}=0\cdot20$. *c*. $u_{max}=0$.

Fig. 4. Comparison of filtrability of serum-albumin dissolved in water and in Hartley's broth. *a*. Solution in Hartley's broth. *b*. Solution in water (interpolated from the data of Fig. 3). (Membrane A.P.D. $20m\mu$.)

Time did not permit a correspondingly complete series of end-point curves to be obtained for filtration of serum-albumin when dissolved in 1 : 1 dilutions in water of stock broth, but comparison was made of systems at different p_H values with one grade of membrane. The values of u_{max} are plotted against the p_H in Fig. 4, together with the corresponding curve for solutions in distilled water, interpolated from the data of Fig. 3.

Results. The high peak in Fig. 3, at which membranes of A.P.D. $45m\mu$ completely retain serum-albumin in aqueous solution corresponds fairly closely to its isoelectric point, p_H 4·88 [Svedberg, 1930]. It is significant that the adsorption of the protein on collodion particles as a function of the p_H exhibits a high maximum at the same point [Elford, 1933; Ettisch *et al.*, 1930]. Stock broth improves the filtration at all p_H values but most markedly at the isoelectric point and near it on the alkaline side. The minimum filtrability apparently occurs at the same p_H as in distilled water solution. At the optimum p_H values for filtration in aqueous solution, 3·8 and 8, the end-point is about $11m\mu$. The former p_H is just outside the zone within which Svedberg and Sjögren [1930] show serum-albumin to be monodisperse (p_H 4 to 9).

Serum pseudoglobulin.

Experimental. A complete set of end-point curves was established for filtrations of pseudoglobulin, concentration 0·4 %–0·5 % in 1 % saline, at different values of p_H ranging from 2·5 to 8. The values of $j_{0\cdot5}$, $j_{0\cdot2}$ and j_e, obtained as for

the albumin, are plotted against the p_H in Fig. 5. A comparison of filtrations in broth at different p_H values, similar to that performed for the albumin, is shown in Fig. 6.

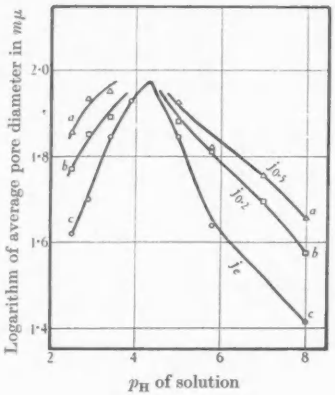

Fig. 5.

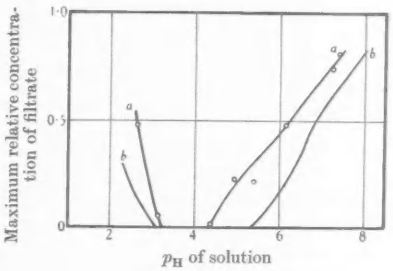

Fig. 6.

Fig. 5. p_H-filtrability curves of serum-pseudoglobulin dissolved in 1 % saline. *a*. $u_{max}=0{\cdot}50$. *b*. $u_{max}=0{\cdot}20$. *c*. $u_{max}=0$.

Fig. 6. Comparison of filtrability of serum-pseudoglobulin dissolved in 1 % saline and in Hartley's broth. *a*. Solution in Hartley's broth. *b*. Solution in 1 % saline (interpolated from the data of Fig. 5). (Membrane A.P.D. 59 $m\mu$.)

Results. Conditions for the poorest filtrability of pseudoglobulin in 1 % saline obtain at p_H 4·4, where a 90 $m\mu$ membrane completely retains the protein. This reaction is well on the acid side of the isoelectric point (p_H 5·4) in contrast to the close correspondence observed between isoelectric point and point of maximum j_e for the albumin. Adsorption of serum-globulin on collodion particles is greatest over a fairly wide range of p_H in the neighbourhood of the isoelectric point [Ettisch *et al.*, 1930; Elford, unpublished experiments], and the maximum is not sharply defined as in the case of serum-albumin.

Broth is found to improve the filtrability of pseudoglobulin at all p_H values (Fig. 6), but the point of minimum filtrability seems to be displaced still further in the acid direction (p_H 3·7). Since the broth itself exhibits a maximum opalescence at about p_H 3, the minimum filtrability observed at p_H 3·7 may depend upon the resultant stability of the broth colloids and of the pseudoglobulin.

The optimum point for the filtration of pseudoglobulin in 1 % saline, as far as the present investigations extend, is at p_H 8. The corresponding value for the end-point is about 25 $m\mu$.

Effect of neutral salt on filtrability.

Solutions of the albumin containing sodium chloride in concentrations varying from 0·05 % to 2·0 % were filtered at selected p_H values to show how increasing the amount of salt in the medium influenced filtration. The salt concentration producing the maximum effect in each case, together with the actual value of

u_{max}, are shown in Table II. The increase in filtrability due to the salt is least marked with systems of high filtrability (low j_e) in water, and the effect becomes greater as the isoelectric point is approached from the alkaline side.

Table II. *Effect of salt on the filtrability of serum-albumin.*

Concentration of protein 0·5 %.

p_H	Membrane $m\mu$	Concentration of NaCl %	u_{max} in NaCl solution	u_{max} in water	Δ u_{max}	j_e in water $m\mu$
7·2	14·5	1·0*	0·34	0·14	0·20	13
6·8	20	0·8	0·90	0·63	0·27	14
4·8	40	2·0	0·95	0	0·95	45

* Only concentration used.

EFFECT OF CONCENTRATION OF PROTEIN ON FILTRABILITY.

Filtration studies on viruses have shown that the filtrability improves with increasing concentration of virus, particularly as the grade of membrane used approaches the end-point. The higher the virus concentration the more quickly is the adsorbing capacity of the membrane satisfied, so that the virus has the greater chance of appearing in the filtrate before blocking sets in. Therefore experiments have always been performed with suspensions of the highest concentrations obtainable, which, however, in the case of virus correspond to molecular concentrations which, for a protein, would be quite undetectable by the sulphosalicylic acid test. In protein solutions, the molecular concentration may be high enough actually to hinder filtration.

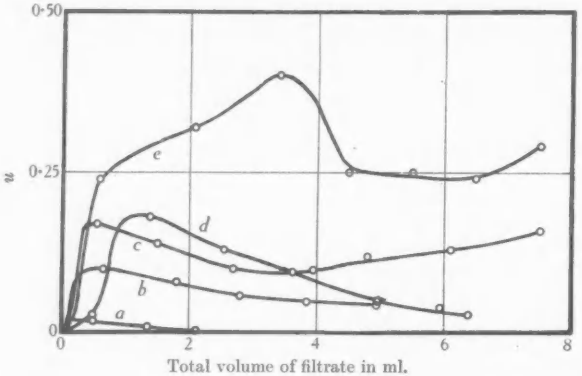

Fig. 7. Filtration curves of serum-albumin at different concentrations. *a*. 4·0 %. *b*. 2·0 %. *c*. 1·0 %. *d*. 0·5 %. *e*. 0·25 %. (Membrane A.P.D. 33$m\mu$; p_H of solution 5·7.)

Experimental. Solutions of serum-albumin at p_H 5·7–5·8 ranging in concentration from 0·25 % to 4 % were filtered through membranes of the same grade. The results are expressed in terms of the relative concentration of filtrate, u, in Fig. 7.

Results. The value of u_{max} decreases with increasing original concentration of the solution filtered. In the systems of highest protein concentration, the primary adsorption on the membrane is most quickly satisfied, but blocking sets in at such an early stage that the first sample of filtrate collected is the most concentrated. Under such conditions it is almost impossible to obtain reproducible results, as the filtrate concentration is rising so rapidly in the first ml. that a very slight variation of the time at which blocking becomes effective will result in widely conflicting values of u_{max}. Hence the concentrations of 4 %, 2 % and probably 1 % are unsuitable for quantitative comparative experiments. On the other hand, a concentration of 0·25 % is rather low for accurate analyses by the refractometer. The usual concentration used in these investigations, 0·5 %, is apparently the most satisfactory.

Effect of pressure on filtrability.

A solution of pseudoglobulin at p_H 5·2 was filtered through membranes of grade 84 $m\mu$ at pressures of 3, 2 and 1 atmospheres and under its own slight hydrostatic pressure in the filter. In the last case, the conditions approximated to those of a diffusion experiment. Fig. 8 demonstrates the effect of increasing

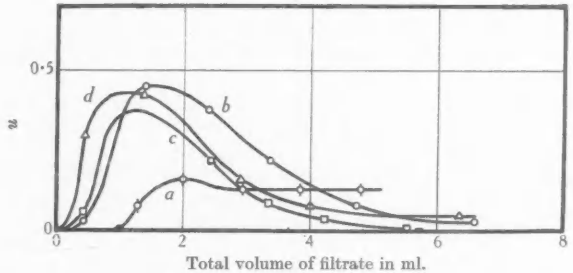

Fig. 8. Filtration curves of serum-pseudoglobulin at different pressures. *a*. No pressure applied. *b*. 1 atm. pressure. *c*. 2 atm. pressure. *d*. 3 atm. pressure. Membrane A.P.D. 84 $m\mu$; p_H of solution 5·2.

pressure in slightly narrowing the adsorption zone [Elford, 1933] and hastening blocking. The range of pressure (1–3 atmospheres) regularly employed in the present studies does not involve much variation in u_{max}.

Discussion.

The p_H-filtrability curves of serum-albumin (Fig. 3) indicate that the p_H range may be divided conveniently into characteristic zones of good and poor filtrability. Between p_H 4·2 and 7, there is a sharp peak of poor filtrability, where the end-point values of average pore diameter lie far above the molecular size (5·4 $m\mu$, disregarding the dissymmetry of the molecule) calculated from ultracentrifugation [Svedberg, 1930; von Mutzenbecher, 1933]. On each side this region grades into a zone of good filtrability, where the curves are relatively flat, and j_e is about twice the molecular size of the protein.

The filtration curves for experiments within the central zone of poor filtrability are invariably of types I, III and IV (Fig. 1). Curves of the other types occur

as this zone merges into the regions of good filtrability; the extreme acid and alkaline systems invariably give curves of types I, II and V. The universality of type I shows a uniform filtration mechanism for membranes which let through the respective systems in nearly undiminished concentration, *i.e.* where u_{max} lies between 0·9 and 1·0. The distribution of the other types indicates two different mechanisms in those filtrations where u_{max} lies between 0 and 0·9.

In a case where the p_H lies within the central zone, the relative concentration of the filtrate drops off at an early stage and decreases as the filtration is continued, eventually reaching zero. The pores have been rendered impermeable to protein, presumably blocked by large deposits of protein within them, and the u_{max} attained is far below the value which might have been reached had this blocking effect not occurred. This process may be termed *abnormal filtration*. On the other hand, when the experiment is made in the extreme acid or alkaline portion of the p_H range, the relative concentration of the filtrate reaches a definite fractional value which is fairly steadily maintained or may even rise gradually as the filtration continues. This is what would be expected for a true sieving mechanism, where the filtrate concentration slowly increases due to the continual gain in concentration of the residue above the membrane [Manegold, 1930] or is maintained constant if the latter effect is offset by a slow blocking. The suggestion of sieving need not necessarily mean that the system filtered is polydisperse. A monodisperse system filtered through a membrane whose pores are not quite uniform in size but have a range of size distribution would exhibit this effect; and our gradocol membranes, while believed to be more uniform than most other types of filters, possess such a range of pore sizes. But regardless of what interpretation is placed upon the filtration curves of types II and V, it is evident that only where these types obtain can the experiments be used for comparing the particle size of the disperse phase with the pore size of the membrane. Hence we propose to term the process which gives rise to these curves *normal filtration*.

The close resemblance in shape of the curve of j_e as a function of p_H for serum-albumin (Fig. 3) to the curve for adsorption of the protein on collodion particles [Elford, 1933] indicates the important correlation of adsorption with filtrability. However, the primary adsorption occurring at the pore surface, whose equilibration takes place within the first ml. or so of filtrate passing and is responsible for the initial steep rise in the filtration curve, is not the decisive phenomenon in filtration. It determines the value of u_{max} only in the case of curves of type I. The significance of the similarity of the curves is probably that the high peak in adsorption in the isoelectric region is due to the same process which causes the blocking of pores in abnormal filtration. That process may be a multiple layer adsorption.

The position and shape of the adsorption peak are the same whether the adsorbent is collodion, quartz, kieselguhr or kaolin (Elford, unpublished experiments), suggesting that the peak is due to adsorption not of protein on adsorbent only but of protein on protein, *i.e.* to the formation of secondary adsorbed layers. This corresponds with the tendency to aggregate which prevails in isoelectric protein solutions, as demonstrated by maximum opalescence and a minimum of the "alcohol number" defined by Fenn [1918] at this point. The blocking in the zone of abnormal filtration is then easily explained. When the protein system is forced through the membrane, multiple layers are adsorbed on the sides of the pores; the latter are narrowed and, as the filtration continues, eventually become blocked. The conditions within the pores are exceptionally favourable for formation of protein aggregates and precipitation, owing to the frequency of

collisions in the limited space. On the other hand, in the zones of normal filtration (which may be termed the stable[1] zones), adsorption of the protein is probably unimolecular. The pores are lined with one layer of protein, and other protein molecules slip through without being retained.

The range of p_H values investigated for pseudoglobulin seems to lie entirely within its abnormal filtration zone (Fig. 5), as the end-point at the extreme alkaline side is still more than three times the size of the molecule (6·9 $m\mu$, disregarding molecular dissymmetry). This is confirmed by the filtration curves, which are nearly all of the types I, III and IV. The broad zone of abnormal filtration is quite in agreement with the fact that solutions of pseudoglobulin are generally less stable and more apt to aggregate and precipitate than those of albumin. In solutions whose p_H values lie in the centre of this zone, even the adsorption on the top surface of the membrane apparently suffices to initiate a progressive precipitation. The residue above the membrane after filtration of such a system usually contains a copious precipitate of protein, and the concentration of the supernatant solution, in spite of a decrease in volume of ten- or twenty-fold, is less than that of the stock solution originally introduced into the filter. The adsorption-p_H curve of the pseudoglobulin, again, closely resembles the filtrability curves.

The general effect of salt in improving filtrability, observed at the isoelectric point and on the alkaline side of it, may now be interpreted as a peptising action due perhaps to alteration in charge and hydration of the protein molecule by adsorption of ions. It suppresses aggregation and formation of multiple adsorbed layers and renders filtration less "abnormal." The more abnormal the filtration is in the absence of salt, the more the presence of salt would be expected to affect it; and this is found to be the case (Table II). This is further illustrated by the fact that saline medium improves the filtrability of pseudoglobulin more than that of albumin, solutions of the former protein being characterised by their relative instability and abnormal filtration. The stabilising effect of salt on the globulin is also apparent in decreasing visible opalescence and increasing the "alcohol number." An increase in the electrolyte content of the system will also progressively reduce the Donnan potential at the surface of the protein particle and thus tend to minimise any consequent electro-viscous effects.

The extra-digested broth probably peptises the protein in much the same way as does the sodium chloride, owing to its high content of salts and amino-acids (Table I). The other broths, however, have an additional effect, and this is probably "lubrication" due to preferential adsorption of a capillary-active constituent on the surface of the membrane pores [Elford, 1933], and consequent suppression, not only of multiple-layer adsorption, but even of unimolecular adsorption of the protein, thereby making conditions the most favourable possible for filtration.

Stock broth can thus improve the filtrability of albumin even at a p_H at which filtration in distilled water solution is normal. Fig. 4 demonstrates that the filtrability is, in fact, increased by broth over the entire p_H range, and the zone of abnormal filtration is much narrower (p_H 4·3–5·5) than for aqueous solutions of the protein (p_H 4·2–7).

[1] Our use of this term should not be confused with that of Svedberg, whose "p_H-stability ranges" refer to stability of monodispersion, with respect to breakdown (occasionally aggregation) of protein molecules. "Stability" is used in this paper with respect to potential aggregation and precipitation.

Optimum conditions for filtration.

It is essential in determining the filtration "end-point" for estimating the particle size of a disperse system to ascertain and utilise the optimum conditions for filtration. These obtain when the filtration is "normal," and when the end-point value is as close to the actual particle size as possible. The fact that a protein in solution, as found by Svedberg in his ultracentrifugation studies, is uniformly monodisperse only within certain definite characteristic ranges of p_H, makes it necessary to confine filtration within these prescribed limits. In the studies with virus suspensions also, a restriction upon the permissible working conditions is imposed by the p_H range of viability in each instance. The evidence presented in the foregoing sections clearly indicates that for the serum-proteins the requirements for optimum filtration conditions would be fulfilled by adopting Hartley's broth, at p_H 8, as the standard suspension medium. This medium at p_H 7·4–7·6 has been consistently used in ultrafiltration analyses of virus and bacteriophage suspensions in this laboratory. Experience has shown that both virus and phages filter under these conditions much more readily than when contained in saline or Ringer solution [Galloway and Elford, 1931; Elford and Andrewes, 1932]. Further, their filtrability in broth is not appreciably affected by changes of p_H within the range of viability whereas in saline and Ringer the filtrability of the viruses and phages decreases as the p_H is changed in the acid direction. It must be remembered in this connection, however, that when working with viruses it is seldom possible to detect with certainty variations in concentration less than tenfold.

The ratio, particle size/end-point A.P.D., for the serum-proteins.

An empirical correction factor, derived from filtration studies with suspensions of known particle size, has been used in calculating the particle sizes of viruses and bacteriophages from the values of their experimentally determined end-points [Elford, 1933]. The ratio, particle size/end-point A.P.D., was evaluated individually for certain bacteria, gold sols, haemoglobin and egg-albumin. The correction factor was given as ranging from this ratio to a figure about 30 % below it. It is of interest to derive the values of the ratio for the serum-proteins (Table III). The sizes of the protein particles, denoted by $2r$, are calculated from

Table III. *Particle sizes and end-points of the serum-proteins.*

	Medium	Particle size, $2r$ $m\mu$	End-point j_e ($m\mu$)	Ratio $\frac{2r}{j_e}$
Serum-albumin	Water, p_H 8·0	5·4	11	0·49
Serum-albumin	Broth, p_H 7·8	5·4	9–10	0·54–0·60
Serum-pseudoglobulin	Broth, p_H 7·8	6·9	11–12	0·57–0·63

Svedberg's and von Mutzenbecher's values for the molecular weights, neglecting their slight dissymmetry and regarding them as spherical. The end-points under the optimum conditions, *i.e.* solution in Hartley's broth at p_H 7·8, were obtained by extrapolation from the lower portions of the end-point curves of the respective systems. The end-point for serum-albumin in water at p_H 8·0 is from the data of Fig. 3.

The end-point for the albumin in water is naturally higher than in broth, even though the filtration is quite normal in both cases, because of the additional "lubrication" afforded by the latter medium. The ratios $\frac{2r}{j_e}$ for the two proteins

in broth agree satisfactorily with the value (0·54) for haemoglobin in the same medium [Elford, 1933].

It is hoped to present later similar data for proteins of still larger molecules, when a general tabulation of the values of the ratios will be made.

Filtration of artificial mixtures.

Examination of the respective end-point curves showed that at p_H 3·8 membranes of A.P.D. 40 $m\mu$ to 80 $m\mu$ retain pseudoglobulin (in saline) completely, whereas albumin (in distilled water) passes them in nearly undiminished concentration. To determine the behaviour of a mixture of the two, a solution at p_H 3·8 was made up to 0·5 % in respect to each protein in 1 % saline and filtered through several grades of membranes in this range. The results are shown in Table IV.

Table IV. *Filtration of artificial mixtures.*

0·5 % albumin + 0·5 % pseudoglobulin in 1 % NaCl p_H 3·8.

Membrane A.P.D. $m\mu$	Pseudoglobulin (1/2 sat. $(NH_4)_2SO_4$)	Albumin (sulphosalicylic acid)
70	Positive	Positive
55	Trace	Trace
40	Negative	Negative

Under these conditions, the filtrability of the pseudoglobulin is increased, and that of the albumin is greatly decreased. The improvement in the case of the former protein is probably the result of a stabilising influence exerted by the albumin. On the other hand, the drop in filtrability of the albumin no doubt means that the pores, clogged by multiple adsorbed layers of pseudoglobulin (here undergoing abnormal filtration), are blocked to both proteins equally. Hence the data do not necessarily indicate formation of an albumin-globulin complex. (The mixture is not strictly comparable with the pure solution of albumin, owing to the presence of the sodium chloride in the former; but any effect due to the salt would be small compared with the great decrease in filtrability observed).

Filtration of horse serum.

In order to compare the filtration end-points of serum-albumin and globulin as determined in pure solutions and in their native state in serum, reference is made to the work of Elford, Grabar and Fischer. Their experiments, details of which will be published later, yield the end-point values for the proteins in undiluted serum and in dilutions in broth and in saline (Table V).

Table V. *End-points of the serum-proteins in native serum.*

Pressure 1–3 atm.

	p_H	End-point of albumin $m\mu$	End-point of globulin $m\mu$
Undiluted serum	8·2	9–10	11
Serum: broth, 1 : 9	7·6–7·8	9–10	11
Serum: saline, 1 : 9	7·8–8·0	11	13

It is seen by inspection of Tables III and V that the filtrability of each serum-protein is characterised by the same end-point whether the system filtered is normal serum or the pure solution of the protein in Hartley's broth at p_H 7·8.

This indicates that serum-albumin and globulin exist largely as individual molecular species in their native state in serum, a conclusion also reached by Svedberg and von Mutzenbecher in their ultracentrifugation studies.

Summary.

1. The filtration curve, end-point curve and a quantitative measure of filtrability are defined.

2. The influence of the nature of solvent medium, p_H, neutral salts, concentration of protein and filtration pressure on the ultrafiltration of serum-albumin and pseudoglobulin through graded collodion membranes is described.

3. These effects demonstrate two types of filtration, termed normal and abnormal, and are interpreted in terms of adsorption of protein within the membrane pores.

4. Hartley's broth at p_H 7·6–7·8 is found to be the best solvent medium for filtration experiments with the proteins, as previously noted for bacteriophages and animal viruses.

5. The ratios, particle size/end-point A.P.D., are given for the two proteins for optimum filtration conditions.

6. The filtration end-point of each protein is the same in pure solution in Hartley's broth at p_H 7·8 as in native horse serum.

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XCI. THE REVERSIBLE ENZYMIC OXIDATION OF VITAMIN C.

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Some years ago during the progress of an inquiry on the production of an antiscorbutically active cider from Bramley's Seedling apples it was observed that the juice expressed from the fruit while retaining a very high proportion of its antiscorbutic activity did not reduce indophenol. At that time the writer held the view that the capacity for reducing this indicator was mainly due to a substance which, although closely associated with the vitamin in a protective way, was not the active principle itself. The probability of the production of a reversibly oxidised active form of the vitamin was not at that time seriously considered by him.

This problem was reinvestigated recently in view of the fact which has now been established that *l*-ascorbic acid possesses antiscorbutic activity *per se* and that when this compound is reversibly oxidised [Szent-Györgyi, 1928] it retains its activity [Tillmans *et al.*, 1932; Hirst and Zilva, 1933].

It was found again that juice expressed from Bramley's Seedling apples although unable to reduce indophenol was antiscorbutically active. Thus a daily dose of 3 ml. of the non-reducing apple juice afforded protection to guinea-pigs equivalent to 0·25–0·5 mg. of ascorbic acid or to rather less than 1 ml. of decitrated lemon juice (10 I.U.). This potency is not very much less than that obtained with intact tissue of these apples (usually about 15 I.U. per 3 g.). The apple juice, it was further found, reduced indophenol immediately after expression but rapidly lost this power of reduction. Aqueous extracts prepared with or without cyanide, unlike alcoholic, trichloroacetic acid and strongly acid extracts did not reduce indophenol. Furthermore, the non-reducing apple juice after treatment with hydrogen sulphide regained the property of reducing indophenol. This evidence is very suggestive of a case of enzymic reversible oxidation. Experiments will here be described which strongly support this hypothesis.

Experimental.

The oxidation of vitamin C in lemon juice.

15 ml. of freshly expressed apple juice (Bramley's Seedling), which did not reduce indophenol, were added (*a*) to 50 ml. of decitrated lemon juice p_H 7 (resulting p_H of mixture, 4·4); (*b*) to 50 ml. of decitrated lemon and the mixture adjusted to p_H 3 (reaction of apple juice). These mixtures were allowed to remain in the presence of air at room temperature for 6 hours. At the end of this time the reduction in (*a*) had fallen from 4·9 to 0·8 ml. $N/1000$ indophenol per ml.; in (*b*) from 4·9 to 2·1 ml. of the indicator. The corresponding control solutions of decitrated lemon juice which did not contain any apple juice lost very little of

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their indophenol reducing power. The oxidised solutions were then submitted to reduction with hydrogen sulphide as described by Johnson [1933]. The titre of (*a*) and of (*b*) rose in consequence to 6 ml. *N*/1000 indophenol. These figures were higher than the original (4·9 ml.) owing to the fact that the oxidised apple juice in the samples was also reduced in the process. An experiment performed with pure ascorbic acid in aqueous solution gave similar results.

The biological activity of the reversibly oxidised vitamin C in lemon juice.

Decitrated lemon juice treated daily as above and kept at p_H 4·4 was used in these tests. The indophenol reducing power left in these preparations after they had stood for 6 hours varied with the samples of the apple used. Only very seldom was the reducing power found to have disappeared entirely. Occasionally oxidation was not very advanced after this time. In such cases the preparation was allowed to remain for about 24 hours, after which no reducing power could usually be observed. It was, however, considered inadvisable to test 24-hour samples daily in order to avoid the possibility of considerable direct oxidation by atmospheric oxygen. Daily doses of 1·5 ml. of the oxidised preparations were tested, and decitrated lemon juice of an equivalent reducing capacity was administered to a control group of guinea-pigs. It will be seen from Fig. 1 that the

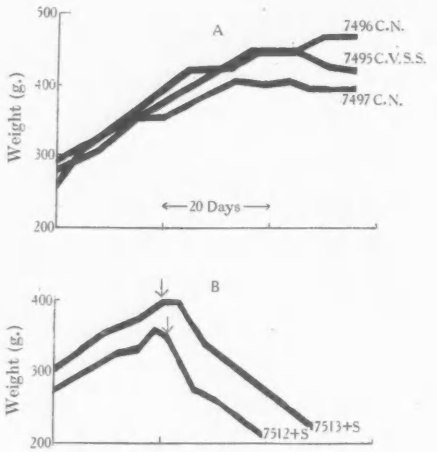

Fig. 1.

C = Chloroformed. V.S.S. = Very slight scurvy.
\+ = Died. N = Normal.
S = Scurvy. ↓ = Onset of clinical symptoms of scurvy.

A. These animals received 1·5 ml. of reversibly oxidised decitrated lemon juice daily. Average reduction value of daily dose 1·4 ml. *N*/1000 indophenol.

B. These animals received as a control untreated decitrated lemon juice. Average reduction value of daily dose 1·8 ml. *N*/1000 indophenol.

reversibly oxidised dose of 1·5 ml. which showed an average daily reducing capacity of only 1·4 ml. *N*/1000 indophenol protected the animals to a very marked extent, whilst a dose of decitrated lemon juice of a similar reducing

capacity afforded hardly any protection. As the activity of apple juice present in the oxidised dose was negligible it may be assumed that the reversibly oxidised vitamin of the decitrated lemon juice was mainly responsible for the protection.

The influence of the absence of oxygen on the action of apple juice on vitamin C.

Experiments similar to the above were carried out at p_H 4·4 in an atmosphere of nitrogen. In a typical experiment the solutions were placed in ampoules which were then alternately exhausted and filled with nitrogen three times. These solutions as well as the controls, which were kept in conical flasks in the air, were stored side by side at room temperature for 24 hours. While the reducing power of the control solution fell from 5·5 ml. N/1000 indophenol to nil per ml. that of the anaerobic sample fell only to 4 ml. N/1000 indophenol. The small loss in reducing power in the latter was most probably due to manipulation, and reversible oxidation, if any, was therefore small.

The influence of boiled apple juice on vitamin C.

Experiments similar to the above were performed in which the apple juice used was previously boiled. The following is a typical experiment in which the reducing capacity of the sample containing the boiled apple juice fell at p_H 4·4 from 4·9 to 3·8 ml. N/1000 indophenol per ml. of solution in 6 hours, a fall which was very probably due to atmospheric oxidation. In the sample with the unboiled juice the original titre fell to 0·8 ml. N/1000 indophenol per 1 ml. of solution.

The influence of boiled apple juice with added peroxidase on vitamin C.

The apple juice used in the above experiments is capable of oxidising the usual peroxidase reagents in the presence of hydrogen peroxide. This oxidative activity is destroyed on boiling, and it was of interest to discover whether the addition of peroxidase to boiled apple juice would restore its capacity to oxidise vitamin C. The preceding experiment was therefore repeated with and without the addition of turnip-peroxidase to the boiled apple juice. It will be seen from the following representative experiment that this addition did not restore the oxidising activity of the boiled apple juice. Thus at p_H 4·4 the solution containing the boiled apple juice alone and the solution containing the boiled apple juice *plus* a few drops of turnip-peroxidase solution, as well as the control decitrated lemon juice containing no apple juice, fell in their reducing capacity from 5·1 to 4·5 ml. N/1000 indophenol per ml. of the solution.

The influence of cyanide on the action of apple juice on vitamin C.

As cyanide inhibits peroxidase activity, an endeavour was made to ascertain whether this reagent would also inhibit the oxidising activity of the apple juice. In these experiments carried out at p_H 4·4 the usual quantities of decitrated lemon juice and apple juice were used. To one sample sodium cyanide was added to make the concentration M/500. This concentration inhibited the peroxidase activity in the solution. The following is a typical example. In the sample containing cyanide the indophenol reducing capacity fell from 5·3 to 1·8 ml. N/1000 indophenol per ml. in 6 hours. In the control sample which did not contain any cyanide the reducing capacity fell to 1·1 ml. in the same time. This concentration of cyanide, therefore, had no serious inhibiting effect on the oxidation of the vitamin. Only when the concentration was raised ten- or twenty-fold was a marked inhibition observed.

Discussion.

The experiments described supply fairly convincing evidence that the apple contains an enzyme which is capable of reversibly oxidising vitamin C without affecting its antiscorbutic potency to any very marked extent. The facts that peroxidase when added to boiled apple juice is incapable of oxidising the vitamin and that quantities of cyanide which inhibit this enzyme do not seriously interfere with the oxidation of the reduced form of the active principle, show that this oxidation is not brought about by peroxidase either alone or as one of the enzymes in a coupled system. The oxidising enzyme seems to resemble the one described by Szent-Györgyi [1931].

This worker found that unboiled, but not boiled, cabbage pulp rapidly lost its capacity for reducing Folin's phenol reagent and for taking up oxygen in a respirometer. Further, the addition of hexuronic acid (ascorbic acid) to cabbage juice greatly increased the uptake of oxygen. He concluded that cabbage contained an enzyme (hexoxidase) which could oxidise reversibly hexuronic acid. The oxidised product obtained by him was not tested for its antiscorbutic activity, as at that time the identity of ascorbic acid with vitamin C was not known. It is, however, quite conceivable that the oxidised product in cabbage juice is also active.

That the oxidising enzyme functions under physiologically controlled conditions in the tissue of the intact apple as well as in the disrupted fruit is more than probable. In fact, evidence which is very suggestive in this respect has been obtained during the last few years in an enquiry on the vitamin C of the apple by the writer in collaboration with Drs F. Kidd and C. West of the Low Temperature Research Station, Cambridge. The knowledge of the distribution of this enzyme in the plant and animal kingdoms would evidently greatly contribute to the solution of the problem of the function of vitamin C in plant and animal organisms.

Summary.

The apple (Bramley's Seedling) contains a thermolabile enzyme which is capable of reversibly oxidising vitamin C without seriously impairing its antiscorbutic activity.

This enzyme does not function under anaerobic conditions and is only inhibited by very high concentrations of cyanide.

The peroxidase of the apple does not seem to be involved in this oxidation process.

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XCII. THE FORMALDEHYDE-AZO-TEST FOR VITAMIN B_1.

BY HENRY WULFF KINNERSLEY AND RUDOLPH ALBERT PETERS.

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(*Received March 5th, 1934.*)

THERE is some vagueness as to the exact scope of the so-called Pauly reaction, such as is given by histidine with diazotised sulphanilic acid. In the broad sense, it might be considered to characterise any substance reacting in a weak alkaline solution to give the characteristic pink or red colour. In the particular sense however it is often now taken to indicate a substance reacting with the reagents studied in detail by Koessler and Hanke [1919; see also Hunter, 1922; 1925].

Jansen and Donath [1926] stated that their crystalline preparations of vitamin B_1 from rice polishings gave a strong Pauly reaction, *i.e.* gave a red colour when treated with diazotised sulphanilic acid in presence of sodium carbonate. On the other hand, Kinnersley and Peters [1927; 1928] found (*a*) that most of the substances in yeast concentrates of vitamin B_1 giving the Pauly reaction (Koessler and Hanke technique) did not fractionate with the activity and (*b*) that fractions of activity 10γ per unit of B_1 did not contain more than 3 % of substances giving a Pauly reaction similar to that of histidine. Guha and Drummond [1929] also obtained active vitamin B_1 fractions from yeast giving feeble Pauly reactions. In ignorance of the true potency of vitamin B_1 itself, it was impossible to decide whether the final entity was responsible for the trace of pink found. In 1931, at the British Association, it was stated [Peters, 1931] that the trace of pink colour given in the Pauly reaction by active yeast concentrates and by Jansen's crystals could be enhanced by suitable choice of alkalinity, and that it was believed that the active entity gave a Pauly reaction[1]. Shortly afterwards this opinion was confirmed in further work, the outcome of which is given in this paper. Meanwhile other researches have given contradictory results, *e.g.* the vitamin B_1 crystals of Ohdake [1932] are stated to give a slow pink diazo-reaction, whereas the crystalline preparations of Tschesche *et al.* [1931] and of Van Veen [1932] gave no Pauly reaction, though the latter author was of the opinion that interaction with diazotised sulphanilic acid took place. Our own crystalline preparations have been stated by us to give an entirely yellow reaction with Pauly's test as performed by Koessler and Hanke, *i.e.* in 2·5 % sodium carbonate. Hence we were correct in attributing our previous 3 % to impurities.

Nevertheless, in essence the opinion as expressed at the British Association Meeting is correct, namely that the vitamin would interact with the diazo-reagent to give a pink colour at a more alkaline reaction than the one employed in the Koessler and Hanke method. We have delayed publication on this question for over two years in the effort to collect conclusive evidence, and we are now presenting shortly some of our data (as a preliminary step). Two circumstances have assisted our progress: (1) the realisation that rather fine shades of alkalinity

[1] It should have been added "in this sense."

would influence the coupling of some substances in the yeast extracts and the degree of yellow rather than pink formed (it is well known that pronounced changes in the alkalinity influence these colours): (2) the discovery of the remarkable effect of formaldehyde.

Test in presence of formaldehyde.

All vitamin B_1 crystalline preparations in our possession interact to produce a pink colour with the following reagent:

N NaOH	100 ml.
$NaHCO_3$	5·76 g.
H_2O	100 ml.

Method of performing test. 0·5 ml. of the diazotised sulphanilic acid[1] (Koessler and Hanke) is added to 1·25 ml. of the reagent in a small test-tube and after 1 minute 1 drop (0·03 ml.) 40 % formaldehyde and then immediately the vitamin in 0·1–0·3 ml. solution of acidity greater than p_H 4·0. A pink colour develops slowly and increases in intensity for 30–60 minutes. After this time it remains approximately constant, though the pink becomes more prominent up to 24 hours owing to fading of the yellow. Standards can be made up with different concentrations of the vitamin from 2 to 6 or 7 pigeon doses and kept for about 14 days. The limit of sensitivity is about 1 vitamin B_1 unit, approximately 1·5–2·0γ crystals. If made up in steps of 1 unit (pigeon dose) in small test-tubes (7 × 1·3 cm.) and corked, reasonably accurate estimates of colour can be made with 5–10γ of vitamin crystals. Trials with a colorimeter have not given as satisfactory results with these dilute solutions as the simple comparative method. The latter can be combined with dilution if desired. With amounts of 1 unit, the colour is ill-defined. Even in the purest specimens of vitamin yet reached, the red is always tinged with yellow. 10γ (5 pigeon doses in our sense) approximately match a mixture of Sørensen's acid and alkaline phosphates (2+8 volumes) of p_H 5 at 15°, containing in 103 ml., 100 ml. of phosphate mixture and 3·0 ml. 0·02 % phenol red.

Influence of formaldehyde.

The N/10 HCl extracts from charcoal [Kinnersley *et al.* 1933]. In the purer preparations of 5γ and under per vitamin B_1 unit, the formaldehyde stabilises the colour. In its absence the colour increases in intensity for a short time and then fades. This perhaps explains Ohdake's [1932] observations. The pink-coloured substance is extracted by butyl alcohol from alkaline solution, and so can often be separated from the yellow substances in impure solutions.

In the less pure preparations up to 100γ per unit the formaldehyde has the effect of suppressing or inducing the gradual fading of the yellow colour caused by impurities (such as histamine), which react with the reagent at the particular alkalinity chosen to give a yellow colour. (At a less alkaline p_H such substances would of course give the usual pink.)

The 50 % alcohol extracts from charcoal and baryta extract of Jansen's acid clay standard. In these cases increase of the formaldehyde to a point at which the vitamin red colour is suppressed is not sufficient to inhibit the yellow colour. Orange colorations are therefore always produced. No attempt has yet been made to explore this further. By use of the Rosenheim-Schuster colorimeter, the red component can be selected and could probably be estimated, but this has not been properly explored.

[1] This reagent will keep for a week, if surrounded by ice in a thermos flask.

In all the impure extracts there is apt to be present in addition a substance giving a colour reaction at a more alkaline p_H. The alkalinity is chosen to reduce to a minimum the influence (*a*) of substances of the histamine type showing pink at more acid reaction and (*b*) of substances showing colours at a more alkaline reaction.

Vitamin B_1 couples at an acid as well as an alkaline reaction. The azo-colour is only produced at an alkaline p_H. The test cannot well be applied at all to extracts less pure than 500γ/unit, as it is obscured by other reactions. In yeast extracts (and other sources investigated), there may be present at the earlier stages (in the extracts of acid charcoal for instance) a substance behaving similarly to the vitamin. It may be distinguished by adding to the pink solution concentrated HCl drop by drop to approximately p_H 8. At this p_H, the colour in question rapidly diminishes, whereas that of the vitamin persists for some time, even at an acid p_H. Beyond this, the test appears to have a fairly high degree of specificity; out of a large number of substances tried, one of the few giving the reaction is acetone, and the colour in this case is not permanent. The question however has not been thoroughly explored. The formaldehyde should be added to an acid solution of the vitamin immediately before the diazotised reagent. An explanation of the action of formaldehyde is not to hand; possibly it is in this case an oxidising action. It has been noticed that methylene blue reduced by sodium hydrosulphite at an acid p_H is not affected by addition of formaldehyde; making alkaline, however, induces much more rapid oxidation of the methylene white in presence than in absence of formaldehyde.

A preliminary exploration with this test has been made of a large number of extracts of seeds, roots, sugars, *etc.*, after treatment with lead acetate and charcoal for the extraction of the vitamin. In many cases there was a substance producing the vitamin type of colour unstable in acid; in no case was there reason to suspect a more active source of the vitamin than yeast. It should be emphasised that in first extracts the concentration of the vitamin is never sufficient to show the reaction.

Table I shows a comparison between vitamin activities by bird and formaldehyde-azo-test; it represents a few only of the numerous estimations made.

Table I.

Amounts (γ) having similar activities (=4 pigeon day doses).

(1) Older preparations

No.	Bird γ	Formaldehyde-azo-test γ
53	10	10
Windaus *et al.*	17·8	18
64·19	10	9·6
56	11·2	13·8

(2) More recent tests

No.	Bird γ	Formaldehyde-azo-test γ
92 A 50	8	8
19	10 (5)*	8·9
Van Veen	8 (10)†	6·8
93 A	10 (7)	12·8

(3) Tests during an early fractionation of a phosphotungstate fraction, p_H 5·5–4·8. p_H refers to the p_H at which fractions were separated.

p_H	Bird (γ)	Formaldehyde-azo-test (γ)
5·3+	51·5 (5)	51·5
5·2+	125 (4)	120
5·0−	157 (4)	230

* Figures in brackets refer to numbers of birds in tests. For probable errors see paper by Kinnersley *et al.* [1933], and for comparison with catatorulin tests Passmore *et al.* [1933].

Note. 1 pigeon day dose = approximately 1 vitamin B_1 unit.

† This is not the preparation recently mentioned in *Nature*.

Tests upon the preparations of Tschesche *et al.*, and Van Veen[1] are also included. The latter is from rice polishings, so that the reaction is given by oryzanin as well as by torulin.

There is no question from an examination of Table I of the close parallelism between the fractionation of vitamin B_1 and the substance in the extract giving the formaldehyde-azo-test. We are clear, however, that we have had in our hands occasionally forms of the vitamin which do not give the test at the same intensity as the bird test. Hence, though it can be said that with the above exception all substances giving this test in these extracts contain vitamin B_1, it is apparently not true that all forms of vitamin B_1 give the test.

Use of the test in fractionation.

The formaldehyde-azo-test is of the greatest value in the fractionation and has enabled us almost to dispense with the animal tests in fractionation of the phosphotungstate extract from the $N/10$ HCl extracts of the charcoal [Kinnersley *et al.* 1933]. By carrying out simultaneous tests with and without formaldehyde during the fractionation at p_H 4, it is possible to separate almost exactly all the vitamin from the substance which gives the yellow reaction, mostly destroyed by formaldehyde under our conditions[2]. Until it is quite certain that pure vitamin B_1 has been obtained, it cannot be proved that the formaldehyde-azo-test is one of its properties. But it can be definitely asserted that the test is of the greatest value in following vitamin B_1 up to a highly potent crystalline stage. This assertion is based upon a large number of experiments over a period of two years. It is possible that the discrepancies still shown in Table I are not due to experimental error (*e.g.* 93 A); this should be decided by work in progress in collaboration with J. R. O'Brien.

SUMMARY.

An azo-test is described of high specificity for vitamin B_1. The specificity is due to the suitable choice of alkalinity for coupling and the use of formaldehyde as a stabilising agent.

We are indebted to the Medical Research Council for a personal grant to one of us. We are also grateful to Mr J. R. O'Brien for advice and help.

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[1] We again thank Dr Tschesche and Dr Van Veen for the gift of these preparations.

[2] Without formaldehyde, the pink colour gradually fades.

XCIII. THE ACTION OF GASTRIC JUICE ON BEEF MUSCLE-GLOBULIN, WITH REFERENCE TO ANAEMIA.

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(*Received March 6th, 1934.*)

Pernicious anaemia results from a failure of the supply of healthy red cells to the circulating blood. No way was known of restoring this lost power of regeneration of healthy red cells until Minot and Murphy [1926] observed that by feeding liver to persons suffering from this type of anaemia increased numbers of reticulocytes, or young red blood cells, appeared in the circulation, with the result that the blood was gradually restored to its normal condition.

Following on this discovery, Castle [1929; Castle and Townsend, 1929] announced that changes in the blood, similar to those produced by liver feeding, could be caused by daily administration of raw beef which had previously been digested by normal gastric juice, either in the stomach of a normal person or *in vitro*. Neither beef nor gastric juice was effective alone or when fed successively. Castle suggested that pernicious anaemia is a deficiency disease of a new type; that it is due to a lack of some unknown substance or principle produced from protein during normal digestion; and that the gastric secretion in pernicious anaemia is deficient in a factor necessary for the production of this principle.

According to this work there are, therefore, two factors involved in the maintenance of the proper supply of red blood cells: (1) an intrinsic factor in the gastric juice, and (2) an extrinsic factor contained in protein. By the interaction of these factors the haematopoietic principle is produced. Normally this principle is concentrated in the liver, hence the efficacy of this organ in the treatment of anaemia.

In his later work Castle *et al.* [1930] demonstrated that the protein of beef muscle, precipitated at p_H 6 and washed with saline of the same reaction, could act as the extrinsic factor: washed caseinogen and wheat gluten were unsuitable. The principle could be obtained by digesting this precipitated beef protein with normal gastric juice at a neutral reaction. The intrinsic factor appears to be neither HCl, nor pepsin, nor trypsin; it is confined to the gastric juice and is destroyed by boiling and behaves like an enzyme [Castle *et al.*, 1931].

In view of what is known of the intrinsic factor, its resemblance to an enzyme, *etc.*, we felt that it would be of value to study more closely the interaction of gastric juice and muscle-protein with the object of obtaining chemical evidence of the production of the active principle. Up to this time the test for the presence of the haematopoietic principle in digests, and incidentally, for the presence or absence of the intrinsic factor in a given sample of gastric juice, has been a reticulocyte response after feeding the products of digestion to cases of pernicious anaemia.

There is evidence that the active principle produced during the digestion of muscle-protein by gastric juice is a water-soluble nitrogenous base, not precipitated by trichloroacetic acid [Cohn *et al.*, 1930]. We therefore planned to determine whether a non-protein nitrogenous body results from such digestion by normal gastric juice *in vitro* near the neutral point, and if so, whether the gastric secretion of cases of pernicious anaemia shows a diminished activity in this respect.

Experimental.

Gastric juice (obtained 1 hour after subcutaneous injection of histamine, or following ingestion of 5 % alcohol) was incubated with muscle-protein in a buffered medium of approximately p_H 6. The unchanged protein was removed by means of trichloroacetic acid and the N in the filtrate determined.

A difficulty which we early encountered was the occasional presence of minute amounts of trypsin in the gastric juice: such tryptic activity was exceedingly small but was sufficient to cast doubt upon our findings. Experiments with commercial trypsin (Liquor Pancreaticus (Armour) precipitated with alcohol, the precipitate washed with alcohol, dried and taken up in saline) showed, however, that the amount of soluble N produced from beef-muscle by this enzyme is 50 % less at p_H 6 than at p_H 8. We were able, therefore, by determining the extent of digestion at p_H 8, to make a suitable correction for tryptic activity when it was present. In doing this we were obviously neglecting the possibility of digestion such as we were seeking occurring at the alkaline reaction, a circumstance which could not be excluded and which, in any case, does not minimise the significance of our findings.

The activity of pepsin under the conditions of our experiments becomes negligible at p_H 3·5, *i.e.* well removed from the region in which we were working.

Method. The gastric juice was freed from solid particles and its reaction adjusted to p_H 6 with alkali or acid as required. 5 ml. were measured into each of three test-tubes: to one of these tubes were added 5 ml. of $N/5$ HCl; to the others 5 ml. of $M/20$ phosphate buffer solution of p_H 6 and 8 respectively. The contents of the tubes were mixed and the reaction checked with the glass electrode. 4 ml. portions of this mixture were separately transferred to test-tubes containing 50 mg. of muscle-protein (this was probably the globulin of muscle and was prepared according to the directions of Castle *et al.* [1931], dried and powdered). The remaining fluid was heated to the boiling-point, cooled, and 4 ml. portions were similarly put up with protein as controls. All the tubes were incubated at 40° for 3 hours and gently agitated 3 or 4 times an hour. When incubation was complete 2 ml. of 25 % trichloroacetic acid were added to each tube, and the tubes after shaking were allowed to stand overnight. Finally, the contents of the tubes were filtered and the N in 4 ml. of the filtrates determined by the micro-Kjeldahl method, using $N/100$ acid. The results represent the differences between the N values of the two digests, calculated as for 100 ml. of gastric juice.

Results.

In Table I our findings in a series of experiments on the gastric juice from normal and pernicious anaemia and other patients are set out. Where the samples of juice contained trypsin, as evidenced by digestion at p_H 8, the amounts of soluble N produced at p_H 6 have been corrected by subtraction of one-half the values obtained at p_H 8.

It will be seen that in all but two cases in the normal group, N amounting to 5–17 mg. per 100 ml. of juice was produced at p_H 6. There is no apparent reason

Table I. *Action of gastric juice on muscle-protein.*

mg. of soluble N per 100 ml. of juice.
(Values at p_H 6 are corrected for trypsin.)

	p_H 1–2	p_H 6	p_H 8
Normals	146	14	9
	227	17	34
	234	13	0
	164	17	11
	200	0	2
	151	7	1
	157	5	2
	174	7	6
	153	11	0
	192	5	0
	156	6	0
	155	11	0
	160	0	0
Pernicious anaemia	78	0	3
	128	0	5
	31	5	6
	30	4	14
	42	13	23
	24	0	5
	*203	1	0
	61	1	3
	24	0	8
Achlorhydric secondary anaemia	99	22	0
	61	4	0
	66	10	5
	—	6	2
Sub-acute combined degeneration of spinal cord	123	8	6

* Free HCl after histamine.

for the failure of two cases to show digestion, and no explanation is possible at this stage.

Turning now to the findings in the collection of cases of pernicious anaemia, with one exception, digestion at p_H 6 was very considerably less than in the normal group: comparison of the two groups clearly shows a difference in the behaviour of the anaemic and normal secretions in regard to the quantities of soluble N produced under the conditions of the experiments.

Of four cases of achlorhydric secondary anaemia, two conformed to the normal and two to the pernicious type.

The amount of N produced at p_H 6 bears no relation to the magnitude of either tryptic or peptic digestion. The juice in pernicious anaemia was weak in peptic activity under these conditions: this is in agreement with the findings of other workers using different substrates.

One case in the pernicious group was found to have free HCl in the gastric juice after histamine and peptic activity above the normal average. The occurrence of free HCl in the gastric secretion in this disease is extremely rare, and it is particularly interesting that in this case there should have been practically no evidence of activity by our method. The blood of the patient, a female, showed R.B.C., 1,400,000; Hb., 36 %; and C.I., 1·3.

In addition we have made some observations of a preliminary nature on the effect of p_H on the production of the soluble N. Samples of normal gastric juice

were allowed to act upon muscle-protein, in a buffered medium, at various reactions. Two curves are reproduced in Fig. 1. In the same figure are plotted curves for the action of pepsin and trypsin under similar conditions.

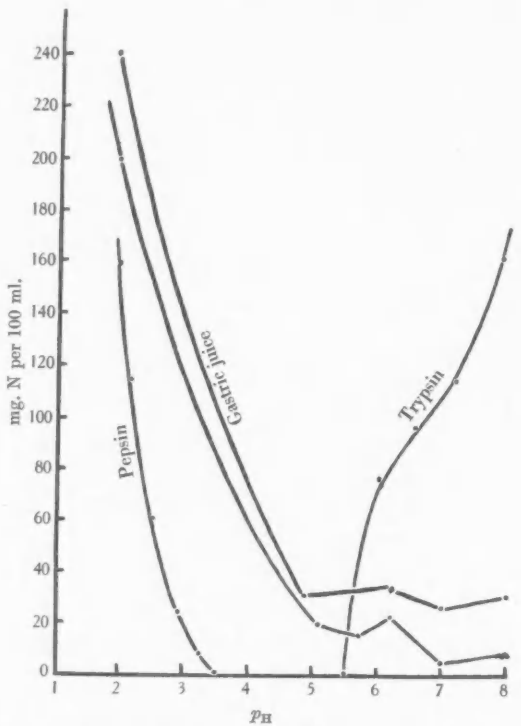

Fig. 1. Action of pepsin, trypsin and gastric juice on muscle-globulin.

Castle *et al.* [1931] found that commercial pepsin was devoid of the intrinsic factor: we assume that the behaviour of the enzyme preparations used by us (pepsin scales, B.P., and Armour's pancreatic extract) is in accordance with that of the gastric enzymes. It will be observed that just as the action of pepsin becomes negligible at p_H 3·5, trypsin ceases to produce any N from muscle-protein at about p_H 5·5, with the result that there exists a range from p_H 3·5 to 5·5 over which neither enzyme yields demonstrable amounts of N under the conditions of our experiments. In regard to gastric juice, however, it is clear that even at reactions within the range p_H 3·5–5·5 definite activity exists, and it would seem to be an advantage in future study to concentrate on this region and so eliminate altogether the participation of pepsin and trypsin.

At this stage we can do little more than record these findings in the hope that subsequent work will reveal their true significance.

We have had in mind the possibility that a bacterial agency might be responsible for the effects obtained. Control experiments in which toluene was

used to inhibit bacterial activity still gave positive results. Further, it is in the anaemias in which one finds a rich bacterial flora in the stomach owing to the absence of free acid that the low results were obtained.

If, as is possible, the nitrogenous substance produced in our experiments during digestion of muscle-protein by gastric juice near the neutral point is related to the haematopoietic principle of Castle, then we have demonstrated in a direct manner the relative absence of the intrinsic factor from the gastric secretion in pernicious anaemia. In consideration of the finding of a certain degree of activity among the anaemias, it must be remembered that even if the gastric juice in pernicious anaemia had all the characteristics of normal juice it is highly probable that its normal functions would be disturbed by a deficiency of secretion, the amount of gastric secretion being so small in this disease. When the secretion is not only scanty in amount but poor in quality it can easily be understood that serious impairment of its function may result.

In regard to the findings in achlorhydric secondary anaemia, it is of much interest, in view of the possibility of a transition from this condition to true pernicious anaemia, that some cases show little activity.

We hope that continuation of this work with improved methods of investigation will lead to a fuller understanding of the significance of Castle's observations and of the rôle of gastric secretion in the workings of the body.

Summary.

1. Small amounts of soluble N are produced when normal gastric juice is incubated with beef muscle-protein at p_H 6.

2. The amounts of this N produced by the gastric secretion from cases of pernicious anaemia were found to be diminished.

3. In the few cases of achlorhydric secondary anaemia studied the results were variable.

4. Pepsin and trypsin play no part in the process.

5. It is possible that the intrinsic factor (Castle) of the gastric juice is concerned in the production of the nitrogenous substance.

It gives me much pleasure to express my thanks to Prof. O. L. V. de Wesselow, Director of the Medical Unit, for suggestions and criticism in the course of this work, and to the Medical Research Council for a part-time grant.

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XCIV. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES.

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Kerr [1929] concluded that in solutions outside the physiological range erythrocytes were permeable to Na^+ and K^+. He also observed that ox erythrocytes suspended in an aqueous solution containing 200 mg. K per 100 ml. showed a greater increase in their K content than did cells suspended in serum of which the K content had been raised to 200 mg. per 100 ml. and hence assumed that normal serum lowered the permeability of erythrocytes. In view of the well recognised depressing effect of Ca^{++} on the permeability of a number of animal membranes, *e.g.* in the case of *Arbacia* eggs as described by Lucke and McCutcheon [1928], it was thought to be not improbable that the serum-Ca played an important rôle in determining the impermeability of the erythrocyte membrane to Na^+ and K^+ which is normally observed. The experiments to be described were carried out primarily to test this theory.

Experimental.

Freshly collected ox blood was filtered through muslin, centrifuged, the serum and top layer of corpuscles were removed and 25 ml. of corpuscles suspended in 25 ml. of saline in a corked flask. After a given time the suspension was centrifuged, the saline removed and 5 ml. of corpuscles measured out into a beaker and diluted with distilled water. The proteins were precipitated with 10 ml. of trichloroacetic acid, filtered off and the filtrate was evaporated to dryness in a silica flask. Ashing was completed by Kutz's method [1931]. The residue was taken up with water and made up to 10 ml.

Sodium determination. The volumetric method of Kahane [1930] was tried but found to be quite unsatisfactory. Balint's [1924] modification of the Kramer-Tisdall method gave good results and was employed throughout.

Potassium determination. The method of Kramer [1920] was used.

Blood-volume changes. These were determined by blood counts on the centrifuged corpuscles and by haematocrit determinations on the suspension.

Results.

In Table I are shown the results of suspending ox corpuscles in various aqueous solutions using this procedure. Exp. 1 shows a large decrease in the potassium content of the corpuscles when suspended in 0·9 % NaCl. Addition of $CaCl_2$ up to 100 mg. of Ca per 100 ml., which is roughly ten times as much as occurs in normal serum, produces no significant effect on this decrease. Exp. 2 shows that there is little difference in K content between the control cells and those suspended in NaCl, Ringer solution and buffered Ringer. Now these two experiments differ in the way in which the controls were determined. In Exp. 1, as

Table I.

No.	Washing fluid	Time	K mg./ 100 ml.	Na mg./ 100 ml.	Vol. chge. %	Corr. K	Corr. Na
1	Serum	—	87·9	—	—	87·9	—
	0·9% NaCl	Overnight	78·9	—	−5	75·0	—
	0·9% NaCl + 20 mg. Ca/100 ml.	,,	79·1	—	,,	75·1	—
	0·9% NaCl + 40 mg. Ca/100 ml.	,,	79·5	—	,,	75·5	—
	0·9% NaCl + 60 mg. Ca/100 ml.	,,	78·9	—	,,	75·0	—
	0·9% NaCl + 100 mg. Ca/100 ml.	,,	80·0	—	,,	76·0	—
2	Serum*	Overnight	58·5	—	+3	60·2	—
	0·9% NaCl	,,	63·0	—	−6·3	59·6	—
	Ringer	,,	67·4	—	−13	58·6	—
	Buffered Ringer	,,	68·5	—	−12	60·3	—
3	Serum*	6 hours	61·1	—	+3·2	63·1	—
	NaCl oxalate	,,	61·8	—	−2·9	60·0	—
	Oxalated serum	,,	64·0	—	−7·3	59·3	—
	Ringer oxalate	,,	64·8	—	−9·4	58·7	—
4	Serum	Overnight	86·1	180	—	86·1	180
	Serum*	,,	77·6	196	12·3	68·0	175
	1·5% KCl	,,	192	155	−6	181	146
	1·5% KCl + $CaCl_2$	,,	198	154	−9	180	140

* Corpuscles left in contact with their serum.

in Kerr's experiments, the control corpuscles were not left in contact with their serum but were immediately ashed and analysed for potassium, whilst in the second experiment the control corpuscles were left suspended in serum for the same period as the others were left in their respective aqueous solutions. If the corpuscles are impermeable to Na and K when in contact with their serum, then there should be no difference between the values obtained by either method. The fact of the small difference between the control corpuscle-potassium and that of corpuscles suspended in NaCl in the one case and the large difference in the other case would point to the possibility of potassium leaking out of the corpuscles almost as well when in contact with serum as when in contact with an aqueous NaCl solution. Exp. 4 of Table I, in which controls were determined in both ways, confirms this, the K content of corpuscles left overnight in contact with serum in the ice-chest decreasing by 18 mg. per 100 ml. Returning to Exp. 2 it is seen that it is very difficult to draw any definite conclusion as to the effect of suspending corpuscles in Ringer or the effect of buffering the solution, since the differences are small and the error introduced by the volume-change correction would probably cover these differences. Again in Exp. 3 it appears that oxalated serum approximates more closely in behaviour to NaCl solution than to normal serum but the differences are small and come within the maximum error. In 1·5% KCl an increase of over 100% in the K content and a decrease of about 30% in the Na content are observed. The Ca^{++} seems not to affect the permeability of the corpuscle membrane to K^+ or Na^+.

It was now felt necessary to develop a technique in which the error introduced by the blood-volume correction, amounting on the average to about 5 %, could be removed. In the next series of experiments 5 ml. of corpuscles were placed in a centrifuge-tube and 10 or 5 ml. of the saline added. After a given period the corpuscles were centrifuged down, and the supernatant fluid was carefully and completely sucked off, great care being taken not to remove any corpuscles. The whole of the corpuscles in the tube were then laked, ashed and analysed for Na and K. This method is independent of the volume changes, as the whole of the corpuscles are analysed and not an aliquot as previously. Table II shows some of

Table II.

No.	Washing fluid	Time hrs.	K mg./ 100 ml.	Na mg./ 100 ml.
1	Serum	—	71·2	—
	Serum*	4	68·3	—
	0·9 % NaCl	4	65·0	—
	Buffered Ringer	4	67·9	—
2	Serum*	3	84·4	182
	1·5 % KCl	3	146	135
	1·5 % KCl + $CaCl_2$	3	145	136
3	Serum	—	64·7	190
	Glucose	3	63·1	145
4	Serum	—	75·4	174
	84 % serum; 16 % H_2O	2	67·2	136
5	1·5 % KCl	2	151	125
	1·5 % KCl; 0·9 % NaCl	2; 0·5	85·4	163
	1·5 % KCl; 0·9 % NaCl	2; 1	83·7	163
6	Serum	—	—	208
	Glucose	0·5	—	151
	1·5 % KCl	0·5	—	153
	Glucose: KCl, 9 : 1	0·5	—	150

* Corpuscles left in contact with their serum.

the results obtained by this method. Exp. 1 shows that in 0·9 % NaCl alone the corpuscles lose more K than they do in serum, whilst in buffered Ringer the losses are approximately the same. Hence the losses of K in NaCl observed are probably due to a general increase of permeability consequent upon certain changes due to degradation characteristic of dead tissue and not to the removal of any specific factor in the serum.

Losses of Na and K are experienced on suspending the corpuscles in isotonic glucose or dilution of the serum by about 20 %. The large increase in K content and the large decrease in Na content observed on suspending corpuscles in KCl solution might quite reasonably be interpreted as adsorption and desorption phenomena. It may be recalled that the average contents of ox-serum are 20 mg. K and 300 mg. Na per 100 ml. Hence it would be expected that if adsorption occurred at the serum-interface Na would be the chief constituent of this adsorption layer. Thus suspending corpuscles in a medium containing very little or no Na, *e.g.* in glucose or KCl should produce a large drop in their Na content. The succeeding experiments represent attempts to solve this problem.

In Exp. 5 of Table II three lots of corpuscles were suspended for 2 hours in 1·5 % KCl. One lot was then analysed for K whilst the two others were suspended in 0·9 % NaCl. One lot was immediately centrifuged down and the other after half an hour. If the potassium were actually permeating the membrane one might expect to find a greater amount in the lot immediately centrifuged off than in the lot centrifuged half an hour later. The results show no appreciable difference between the two. (It is to be noted that the centrifuging was allowed to continue for half an hour, that time being thought necessary; it was later discovered that from a slightly hypertonic aqueous solution corpuscles could be centrifuged off within 2–3 minutes.) A comparison of the losses of Na in glucose and KCl shows no difference. If the loss were due to desorption one would expect that the desorption would occur more readily in KCl where there are ions to exchange with the adsorbed Na than in glucose. However it must be pointed out that we are not here measuring relative velocities but the amounts

lost in one specified time. In Table III the results of a series of kinetic experiments are shown. Exps. 1 and 2 show the effect of suspending corpuscles for times varying from 8 to 60 minutes in 1·5 % NaCl. As is observed the corpuscle-Na

Table III.

No.	Washing fluid	Time (mins.)	K mg./100 ml.	Na mg./100 ml.
1	Serum	—	83·2	159
	1·48 % NaCl	8	79·7	197
	,,	30	79·6	218
	,,	60	76·0	213
2	Serum	—	77·5	185
	1·53 % NaCl	7	74·2	232
	,,	15	74·2	247
	,,	22	74·0	255
	,,	26·5	73·8	278
	,,	31·5	73·8	276
3	Serum	—	93·7	176
	1·5 % KCl	4·5	261	131
	,,	7·5	265	129
	,,	15·5	271	126
	,,	30·0	288	126
	1·5 % KCl; 0·9 % NaCl	30; 5	148	200
	,,	30; 9	138	174
	,,	30; 10	137	188
	,,	30; 31	136	176
4	Serum	—	—	160
	1·5 % NaCl	8·5	—	226
	,,	11·0	—	228
	,,	2·85	—	231

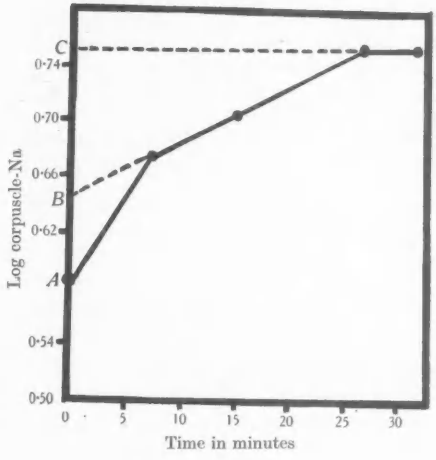

Fig. 1.

increases with time arriving at a stationary value some time between 8 and 30 minutes. The results of Exp. 2 are shown diagrammatically in Fig. 1 where the log of the Na content of the corpuscles is plotted against time. If the process is one of permeation a straight line should result; the graph shows a sharp initial

rise and then a slower one to equilibrium. Exp. 3 shows similar behaviour in 1·5 % KCl and subsequent suspension in 0·9 % NaCl.

Two explanations of Fig. 1 are possible. Either (*a*) the initial sharp increase is due to adsorption, and the slower increase due to penetration, in which case the adsorption would be represented by *AB* and the permeation by *BC*; or (*b*) the effect may be due to inefficient stirring, in which case the whole increase might be due to adsorption or permeation or both. To test this last hypothesis Exp. 4 was performed using twice as much saline for suspending the corpuscles as in Exps. 1, 2 and 3, and the suspension was vigorously stirred for at least 2 minutes before being centrifuged. As the Table shows the phenomenon of the secondary increase has disappeared under these conditions.

In Table IV and Fig. 2 are shown the results of suspending corpuscles in mixtures of NaCl and KCl, the K varying in concentration from 40 to

Table IV.

Washing fluid		Corpuscles	
Na mg./100 ml.	K mg./100 ml.	K mg./100 ml.	Na mg./100 ml.
Serum		84·8	203
39·2	439·5	85·7	207
58·8	427·7	86·1	207
78·4	217·5	89·3	207
117·6	393·0	96·0	207
176·6	358·0	105·0	205
225·0	324·0	113·0	197
298·0	287·0	118·0	189
588·0	116·0	145·0	154

Corpuscles suspended for 1 hour in washing fluid.

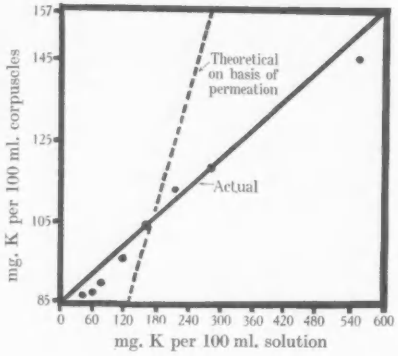

Fig. 2.

600 mg./100 ml. and Na from 440 to 136 mg./100 ml. The total molar concentration of the two ions Na^+ and K^+ was kept constant to maintain identical tonicity of the different solutions and so prevent differences in volume.

Applying the Freundlich adsorption equation:

$$c = kC^{1/n}$$

this may be written:

$$\frac{kC}{C^{\left(\frac{n-1}{n}\right)}} = c,$$

the denominator of the left-hand side representing the factor introduced to account for the limited area of the surface available for adsorption. Applying this to the case where we have both Na^+ and K^+ present:

$$c_{K^+} = \frac{kC_{K^+}}{(C_{K^+}+C_{Na^+})^{\frac{n-1}{1}}},$$

assuming that n is the same for both Na^+ and K^+. Now under the conditions of the experiment $(C_{K^+}+C_{Na^+})$ is constant, so that the adsorption isotherm of either component should be a straight line. It is unfortunate therefore that the shape of the curve of corpuscle-K against concentration in the aqueous solution will not help to distinguish between adsorption and permeation; however, the slope of the curve will tell us whether anything like a simple equilibrium is being established. The actual curve, which has been drawn as a straight line, is certainly rather ambiguous, and one must hesitate before deciding how to characterise the phenomenon it represents. The theoretical curve, assuming that K^+ permeates and is divided equally between the two media, is seen to be very much steeper than the actual curve, showing that if the increases are due to permeation they must occur in accordance with some very obscure equilibrium.

Discussion.

The serum-factor of Kerr appears not to be Ca^{++}. The apparent difference in behaviour between corpuscles in serum and those in NaCl solution has been shown to be illusory, depending on the method of determining the control. K leaks out of the corpuscles into the serum at about the same rate as into NaCl solution. The results of suspending corpuscles in isotonic glucose and diluted serum do not agree with those of Hamburger [1910]. Hamburger found no change in glucose solution and penetration of Na in diluted serum. A point which bears very strongly against the explanation of the losses of Na in KCl and glucose solutions as being due to desorption is the following. If the total molar concentration of Na+K in the corpuscles is compared with that of the serum we find that they are equal; *e.g.* the average of seven different lots of blood gives a total conc. of 14·9 *mM*/100 ml. for the corpuscles and 14·9 *mM*/100 ml. for serum. This of course is to be expected. Now if we assume that approximately 25 % of the corpuscle-Na is adsorbed on the surface, as Exps. 2, 3, 5 and 6 of Table II would indicate, then the average total molar concentration in the corpuscles becomes 13·7 *mM*/100 ml., *i.e.* there would be an osmotic difference between the cells and serum at equilibrium, which of course is impossible. It must therefore be concluded that the losses of Na in KCl and glucose solutions are due to permeation. Hence we are confronted with the phenomenon of a rapid permeation (within 5 minutes) of some 50 mg. Na per 100 ml. to an equilibrium state in which a high concentration gradient is still maintained. From Table IV it is observed that the concentration of Na in the washing fluid may be decreased from 440 to 360 mg. per 100 ml. without producing any significant change in the corpuscle-Na, which seems to indicate that there is a concentration gradient which has to be exceeded before any permeation can occur.

As far as the K^+ ion is concerned a definite but small loss of K occurs in NaCl solution which must be due to permeation. All that can be said of the behaviour of corpuscles in KCl solution is that if permeation of K^+ does occur it takes place within 5 minutes, and an equilibrium position is reached remotely different from that expected on the basis of free permeation.

A possible explanation of these phenomena might be arrived at on the assumption that treatment of the cells with the solution examined itself affects the permeability of the corpuscles, increasing the proportion of those which may be called "dead cells." These "dead cells" will establish a perfect thermodynamic equilibrium with the surrounding fluid, whilst the remainder will maintain their normal impermeability. The net effect will then appear as a partial permeability such as is actually observed. Against this is the fact that only in rare instances was any haemolysis observed, so that a state of permeability to Na^+ and K^+ must be postulated which lies mid-way between those of the normal cell and the truly "dead" cell. Further, one would have to assume that the corpuscles are much more permeable to Na^+ than to K^+ in this hypothetical dead state, since in glucose solutions only about 2 % of the K^+ is lost compared with about 30 % of the Na^+.

Ponder and Saslow [1930; 1931] conclude from their measurements of corpuscular volume that in isotonic salt solutions a loss of osmotic substances occurs, and in hypertonic NaCl and KCl the two salts penetrate to such an extent as to produce an increase in volume after 5 minutes. The results described in this paper show the loss of only small quantities of K in isotonic NaCl and large quantities of Na in KCl accompanied, however, in the latter case by a larger increase in the K content. In 1·5 % NaCl the increases of Na observed are certainly not sufficient to demand an increase in corpuscular volume, and in actual fact an invariable contraction occurred in 1·5 % NaCl and KCl solutions lasting for hours. It must be borne in mind, however, that Ponder and Saslow were working with rabbit's blood, and such large differences in behaviour between species are not out of the question. Reference may here be made to the work of Woodhouse and Pickworth [1932], who measured the permeability of corpuscles to a large number of ions. These authors did not study the influence of K^+ ions as "it may be taken without further experimental proof that the erythrocyte is impermeable to K^+," yet if they had measured the permeability of K^+ they would have obtained a "permeability coefficient" of over 40, a value they obtained for Cl^- in NH_4Cl. Until some method has been devised which will decide unequivocally whether a given ion permeates or is adsorbed one must accept with reserve their statement that the NH^+_4 ion permeates readily.

Summary.

1. In 0·9 % NaCl ox erythrocytes lose K; this loss is unaffected by the presence of Ca^{++} ions and is about equal to the loss sustained in their normal serum under the same conditions.

2. In 1·5 % KCl the corpuscles lose 25–30 % of their Na within 5 minutes; no further appreciable losses occur after this time. The corpuscle-K increases by 100–200 % in the same time and remains constant.

3. In 1·5 % NaCl a considerable increase in corpuscle-Na occurs, but not sufficient to raise the corpuscle concentration to that of the external solution.

4. In glucose solution a large loss of Na and a small loss of K occur. The loss of Na is the same in glucose as in KCl or in mixtures of the two.

5. In diluted serum losses of Na and K are observed.

6. The corpuscle-K is plotted against the concentration of K in the suspending fluid when the latter is varied between 40 and 600 mg./100 ml. The results are not in accord with what would be expected on the basis of ready permeation and only fit approximately an adsorption curve.

My thanks are due to Prof. J. C. Drummond for his kind interest and helpful criticism, to the Department of Scientific and Industrial Research and the Medical Research Council for personal grants during the tenure of which this work was done, and to the trustees of the Dixon Fund for defraying part of the expenses of the research.

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XCV. CALCIFICATION OF HYPERTROPHIC CARTILAGE *IN VITRO*.

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Introduction.

In a previous paper on the experimental calcification of cartilage *in vitro* [Robison *et al.*, 1930] the conclusion was stated that two distinct mechanisms are concerned in normal calcification of cartilage and bone. The first of these, phosphatase-phosphoric ester, brings about the necessary supersaturation of tissue fluid with bone salt [Robison, 1923; 1932]. The second becomes effective only when supersaturation in sufficient degree has been achieved; it provokes the rapid and orderly deposition of the calcium salt in the ground substance of the tissue [Shipley *et al.*, 1926; Robison, 1926].

The share contributed by each mechanism towards the ultimate deposition of the solid calcium salt may depend on the state of the blood with respect to this salt and on the amount of phosphoric ester available.

The distinction between these two mechanisms is empirical. Their activity becomes apparent under different experimental conditions; but it is not affirmed that the mechanisms are entirely independent.

Previous experiments brought to light the favourable effect of bicarbonate on calcification and the inhibitory effect of other ions, notably Mg^{++}, and of protein, and the inhibition of the second mechanism by potassium cyanide, chloroform or by dehydration of the cartilage by acetone *etc.*

Experiments planned to obtain more information on the nature and properties of the dual calcifying mechanism, and particularly of that component which we have termed the second mechanism, are described in this paper.

Experimental.

The technique was in general the same as that previously employed [Robison *et al.*, 1930]. Longitudinal slices cut from the heads of the long bones of rachitic rats were immersed for periods of 16–20 hours in sterile salt solutions at 37° and p_H 7·4. In these experiments the constant-flow apparatus previously described was not used; but the flasks (20 or 100 ml.) containing the bone slices were slowly and continuously rotated at 37° so that all surfaces of the slices were equally exposed to the calcifying solutions. The slices were subsequently fixed, stained with silver nitrate and cleared in cedar wood oil, so that the amount of new calcification in the zone of hypertrophic cartilage could be more readily judged. This is shown in the tables by a figure (maximum 10) representing the extent of the deposit and bars (maximum 5) representing the density, thus $6\equiv$.

[1] Grocers' Company Research Student. Part of the work described in this paper was included in a thesis submitted by A. H. Rosenheim and approved for the degree of Ph.D. in the University of London.

The composition of the basal solution is given in Table I. It differs slightly from that previously used, approximating still more closely to normal plasma in salt content.

Table I. *Composition of experimental solutions.*

g. per litre basal solution		Ion	Millimols per litre: Experimental solution 8 : 5 : 0	Millimols per litre: Normal plasma
NaCl	6·0	Na	130·0	146·0
$NaHCO_3$	2·2	K	5·6	5·1
KCl	0·3	Ca	2·0	2·5
$MgSO_4, 7H_2O$	0·25	Mg	1·0	1·25
		Cl	108·0	101·0
		HCO_3	26·0	26·7
		HPO_4	1·6	1·0
		SO_4	1·0	2·0

The molar concentrations of the acid and basic radicals in a typical inorganic calcifying solution, 8 : 5 : 0[1], and typical values for normal human plasma [Kramer and Tisdall, 1922] are compared in the same table.

Calcium was added to the basal salt solution as $CaCl_2$ and inorganic phosphate as $NaKHPO_4$.

Comparison of the effects of various phosphoric esters on calcification in vitro.

In view of the rôle ascribed to the phosphatase mechanism experiments were undertaken to compare the effects of different phosphoric esters on calcification *in vitro* with that of the syrupy sodium glycerophosphate (chiefly α-ester) which had been generally used. The effect may be dependent on such factors as the rate of diffusion into, or specific adsorption by, the cartilage and on the rate of hydrolysis by the bone-phosphatase.

In these experiments the period of immersion and the concentration of ester were varied, so that any differences in behaviour might be brought out more clearly. The various esters used and the experimental results are shown in Table II. One rat was used for each experiment.

No very marked differences were observed in the effectiveness of the first five esters in the table. The smaller deposits obtained in 5 hours with hexose-monophosphate may possibly signify that this ester diffuses somewhat more slowly than glycerophosphate into the cartilage, although the calcification produced in 18 hours was equally great with both esters.

In the inorganic solutions, in absence of phosphoric ester, no calcification was obtained in periods up to 6 hours. Diphosphoglycerate, which is one of the phosphoric esters of the red blood corpuscles, was ineffective. This ester was not hydrolysed in separate tests with extracted bone-phosphatase; moreover the calcium salt was so insoluble as to be precipitated in the experimental solutions. It is possible, however, that a monophosphoglycerate is also formed in the red corpuscles, and this being readily hydrolysable would prove a suitable substrate for the bone-enzyme. The question is at present being investigated in this laboratory.

[1] The experimental solutions are referred to in this way throughout the text. Thus solution 8 : 5 : 3 contains 8 mg. Ca, 5 mg. P as inorganic phosphate and 3 mg. P as organic phosphate per 100 ml. basal salt solution.

Table II. *The effect of various phosphoric esters on calcification* in vitro.

mg. per 100 ml. Ca	mg. per 100 ml. P inorg.	Phosphoric ester	Conc. of ester mg. P per 100 ml.	Exp. 1 Time (hours) 18	Exp. 2 Time (hours) 5	Exp. 3 Time (hours) 6	Exp. 4 Time (hours) 4	Exp. 5 Time (hours) 6	Exp. 6 Time (hours) 6	Exp. 7 Time (hours) 18	Exp. 8 Time (hours) 18
8	2	0	0	.	.	.	.	.	.	0	.
8	3	0	0	.	.	.	.	.	.	3=	.
8	4	0	0	8≡	0	0	0	0	.	.	6≡
8	4	α-Glycerophosphate	2	.	.	.	0	.	.	.	.
			3	9≡	.	.	.	0	4=	7≡	.
			4	.	.	8=	.	.	.	.	.
			6	9≡	7= 5=	8≡	5=	.	.	.	.
			10	.	.	.	.	5≡	.	.	.
8	4	β-Glycerophosphate	3	9≡	.	.	.	.	.	.	.
			6	9≡	7≡ 7≡	8≡	.	.	.	.	.
8	4	Hexosemonophosphate (mixed ester of fermentation)	3	8≡	.	.	.	.	.	.	.
			6	9≡	1= 2=	.	.	.	.	.	.
8	4	Fructosediphosphate	4	.	.	6−	.	.	.	.	.
			6	.	.	7≡	.	.	.	.	.
8	4	Trehalosemonophosphate	2	.	.	.	0	.	.	.	.
			6	.	.	.	4−	.	.	.	
8	4	Diphosphoglycerate	3	.	.	.	.	0 *p*	.	.	.
			4	.	.	0	.	.	.	.	.
			10	.	.	.	.	0 *p*	.	.	.
8	4	Lecithin	7	.	.	.	.	.	0	.	.
			15	.	.	.	.	.	0	.	.
			30	.	.	.	.	.	0	.	.
8	2	Sphingomyelin	3	.	.	.	.	.	.	0	.
			8	.	.	.	.	.	.	2=	.
8	0	Pyrophosphate	3	.	.	.	.	.	.	.	0 *p*
			4	.	.	.	.	.	.	.	0 *p*
8	2	Pyrophosphate	1	.	.	.	.	.	.	.	0 *p*

p signifies precipitation in the solution during the experimental period.

Lecithin, which is not hydrolysed by the bone-phosphatase, had no favourable effect on calcification; but on the other hand sphingomyelin[1], which is hydrolysed to a slight extent, had some beneficial effect.

Substitution of pyrophosphate for glycerophosphate yielded negative results, since precipitation of the very insoluble calcium pyrophosphate occurred in solutions containing no more than 1 mg. P per 100 ml. in this form.

Experiments with other esters which might function as substrates for the bone-enzyme are in progress.

Deposition of salts other than those of calcium in the hypertrophic cartilage in vitro.

The object of a further series of experiments was to discover whether the second mechanism of hypertrophic cartilage is specific for the bone salt, the complex carbonato-phosphate of calcium, or whether it can also function for other salts of low solubility. The range of possible salts was restricted by the necessity of obtaining solutions sufficiently supersaturated, yet with total ion concentration not unduly removed from that of the tissue fluids.

[1] We are much indebted to Dr O. Rosenheim, who very kindly provided us with a specimen of sphingomyelin.

Attempts to substitute arsenate for phosphate were unsuccessful owing to the high solubility of calcium arsenate. A solution containing 0·025 *M* calcium and as much as 3 *M* arsenate failed to give any precipitate in 24 hours at p_H 7·4.

The replacement of calcium by other alkaline earth metals was more practicable although the phosphates of these metals are more soluble than those of calcium.

In order to find for these experiments the conditions most comparable with those leading to deposition of calcium salt in the cartilage, preliminary tests were undertaken to establish the lowest concentrations of metal and phosphate at which general precipitation occurred in the salt solutions under the experimental conditions. Concentrations slightly lower than this were chosen for the experiments.

For the above tests and subsequent experiments in this group potassium, magnesium and sulphate were omitted from the basal solution and the bicarbonate content was varied as shown in Table III[1].

Table III. *Deposition of Ca, Ba, Sr and Mg salts in the hypertrophic cartilage* in vitro.

17–20 hours; 37°; p_H 7·4.

Metal	Conc.	g. per 100 ml. $NaHCO_3$	mg. per 100 ml. P inorganic	mg. per 100 ml. P organic	Deposition in cartilage: From inorganic solution	Deposition in cartilage: From solution + phosphoric ester
Ca	0·0025 *M*	0·22	3	0	3 =	.
	,,	0·22	2	1	.	4 ≡
Ba	0·01 *M*	0·6	6	0	0	.
	0·005 *M*	0·6	10	0	0	.
	,,	0·6	14	0	0 *p*	.
	,,	1·0	18	0	0	.
	,,	0·05	0	30	.	6 ≡
	,,	0·05	5	10	.	0
	,,	0·05	5	30	.	7 ≡
Sr	0·01 *M*	0·22	10	0	0 *p*	.
	,,	0·6	4	0	0	.
	,,	0·6	8	0	3 ≡	.
	,,	0·6	12	0	8 ≡	.
	0·005 *M*	0·05	12	0	0	.
	,,	0·6	16	0	0	.
	,,	0·6	18	0	3 ≡	.
	0·0025 *M*	0·6	50	0	4 ≡	.
	0·01 *M*	0·05	0	30	.	5 =
	0·005 *M*	0·05	8	4	.	0
	,,	0·05	8	10	.	7 ≡
	0·0025 *M*	0·05	32	2	.	0
	,,	0·05	32	4	.	4 =
Mg	0·1 *M*	0·05	14	0	0	.
	,,	0·05	16	0	0 *p*	.
	,,	0·05	10	4	.	0
	,,	0·05	10	10	.	4 =
	,,	0·05	10	30	.	10 ≡

p signifies precipitation in the solution during the experimental period.

[1] In the case of barium and strontium it was found that as the bicarbonate concentration was raised, progressively higher concentrations of inorganic phosphate could be reached before general precipitation in the solution occurred.

Deposition of barium, strontium and magnesium salts in the hypertrophic cartilage could not at first be realised by agency of the second mechanism alone, that is by immersing the bone slices in inorganic solutions containing these metals in place of calcium. Suitable conditions were at length established in the case of strontium; but deposits of barium and magnesium salts have not yet been obtained in absence of phosphoric ester, even with the highest degree of supersaturation which can be realised short of general precipitation in the solution. On the other hand, deposits of the salts of barium, strontium and magnesium were readily obtained in the hypertrophic cartilage *in vitro* by agency of the bone-phosphatase in presence of phosphoric ester. Typical results are set out in Table III, which shows the relative concentrations required for deposition.

The appearance of the deposits, stained with silver nitrate[1] can be seen in Plate I, Figs. 1–4. Fig. 1 shows a deposit of magnesium salt obtained in solution 0·1 *M* Mg : 10 : 60. The deposit was formed in the matrix of the cells, entirely filling the zone of uncalcified hypertrophic cartilage between the epiphysis and the diaphysis. In appearance it closely resembled that obtained with calcium salts.

Deposits of barium and strontium were usually similar in appearance though sometimes they were less sharply outlined, and occasionally the barium salt was deposited in isolated clusters of relatively large crystals, as seen in Plate I, Figs. 5 and 6.

Attempts were made to obtain deposits of lithium and beryllium salts in the cartilage, but the relatively high solubility of lithium phosphate and the extreme insolubility of beryllium phosphate, carbonate and hydroxide offered considerable difficulties, and no definite evidence of deposition was obtained.

We conclude from these experiments that the second mechanism is not strictly specific for calcium, but that it undoubtedly functions better for the calcium salt than for the others which have been tested. It may be that calcium alone forms a highly complex carbonato-phosphate of solubility so much lower than that of the simple phosphates and carbonates that solutions not supersaturated with the latter may be greatly supersaturated with respect to the complex salt which is ultimately deposited.

Effect of potassium cyanide, of fat solvents and of desiccation on the calcifying mechanisms.

In our previous paper [Robison *et al.*, 1930] it was stated that potassium cyanide strongly inhibited calcification in inorganic solutions but had no appreciable effect in solutions containing phosphoric ester. Further experiments have made it clear that the inhibition of the second mechanism by potassium cyanide is only partial and that calcification may still occur in the more highly concentrated inorganic solutions, particularly if the original activity of the second mechanism in the bone is high. This is illustrated by the results in Table IV. The calcification of the control bone slices in solution 8 : 5 : 0 shows that the second mechanism activity was low in rats *a* and *b* and much higher in rats *c* and *d*. In presence of 0·001 *M* potassium cyanide, calcification was still obtained in this solution in bones from the latter rats but not in those from rats *a* and *b*. The effect of 0·01 *M* cyanide was not markedly greater than that

[1] The deposits of barium, strontium and magnesium salts in the hypertrophic cartilage were also found to stain almost as well as those of calcium with alizarin S. In gelatin models, deposits of the phosphates of calcium, barium, strontium, magnesium, beryllium and lithium stained deep red with this dye, as was shown for calcium and strontium by Cameron [1930].

Table IV. *The effect of potassium cyanide on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.							Potassium cyanide									
			Control				0·0001 *M*		0·001 *M*				0·01 *M*			
Ca	P inorg.	P org.	*a*	*b*	*c*	*d*	*c*	*d*	*a*	*b*	*c*	*d*	*c*	*d*
8	5	0	1≡	1=	5≡	9≡	2=	6≡	0	0	1–	4≡	2=	2=
8	6	0	.	.	5≡	.	6≡	.	.	.	2=	.	3=	.
8	3	10	.	.	7≡	.	6≡	.	.	.	7≡	.	.	.

of 0·001 *M*. No inhibitory effect was observed in solution 8 : 3 : 10, containing phosphoric ester.

The previous conclusion regarding inactivation of the second mechanism by treatment of the bone slices with acetone, alcohol or chloroform, or by desiccation, probably requires similar modification. It would seem that the second mechanism, though greatly weakened, is not entirely destroyed by such treatment.

Effect of various organic substances on the calcifying mechanisms.

Calcification both in presence and absence of ester was strongly inhibited by 0·5 % phenol and by 0·3 % thymol; but 0·1 % phenol had little apparent effect. Neither ethylurethane in 1 % solution nor guanidine in 0·1 % solution appeared to influence the calcifying mechanisms.

Inactivation of the calcifying mechanisms by heat[1].

Experiments were undertaken to determine the effect on the two calcifying mechanisms of heating the bone slices at different temperatures. The slices were placed in bicarbonate-free solution 8 : 2 : 0 and maintained at temperatures between 45° and 60° for periods of 10, 15 and 20 minutes. The effect on the two mechanisms was judged by the subsequent calcification of the slices in solutions 8 : 5 : 0 and 8 : 3 : 10, the deposits being compared with those in unheated control slices. No effect on either mechanism was observed after 20 minutes at 45°; but some reduction in the activity of the second mechanism and probably also in that of the phosphatase was apparent after 15 minutes at 48°. The loss of activity was greater at 50° and 55°; but the results were too irregular to justify any conclusion as to the relative rates of destruction. Both mechanisms, however, appeared to be inactivated in 10 minutes at 60°.

Inactivation of the calcifying mechanisms by acid and alkali.

Unsuccessful attempts were made to inactivate the two mechanisms differentially by soaking bone slices for one hour in isotonic salt solutions at different p_H levels. Little effect on either mechanism was noted between p_H 5·4 and 8·9 at 37°; but both were inactivated, at least partially, at p_H 5.

In 1 % ammonia both mechanisms were destroyed in one hour at room temperature. It may be added that preparations of bone-phosphatase are very rapidly inactivated at p_H values below 5·2 or above 10. It is not known to what extent the tissue may be able to maintain an internal p_H differing from that in the surrounding medium.

[1] Other experiments bearing on this question and those discussed in the following two sections have been carried out by Niven and Robison using embryonic rabbit femora. The results are in course of publication.

Survival of the calcifying mechanisms in excised bone slices under various conditions.

The question of the survival of the calcifying mechanisms in cartilage after excision from the body and immersion in various solutions was investigated on account of its theoretical interest and its bearing on the interpretation of experimental results. Some typical experiments are summarised in Table V. The control (0 hour) bone slices from each rat were placed directly in the calcifying solutions 8 : 5 : 0 and 8 : 3 : 10 at 37° and left there for 18 hours. The other slices were first immersed for varying periods in salt solutions, or distilled water, at 0°, 20° or 37° as specified in Table V. They were then transferred to the calcifying solutions for an 18-hour period at 37°.

Table V. *Gradual inactivation of the calcifying mechanisms in bone slices immersed in various solutions at different temperatures.*

Preliminary immersion				Subsequent calcifying solution	Duration of preliminary immersion (hours)						
Rat	Solution	$NaHCO_3$ %	Temp. °C.		0	½	1	2	4	24	48
					Subsequent calcification in 18 hours; 37°; p_H 7·4						
a	A	0·22	0	8 : 5 : 0	7≡	.	.	.	.	2≡	0
				8 : 3 : 10	7≡	.	.	.	.	3≡	0
b	B_1	0·03	,,	8 : 5 : 0	7≡	.	.	.	.	2=	0
				8 : 3 : 10	7≡	.	.	.	.	3≡	0
c	A	0·22	20	8 : 5 : 0	8≡	7≡	.	6≡	5≡	.	.
				8 : 3 : 10	3≡	.	.	.	9≡	.	.
	,,	,,	37	8 : 5 : 0	8≡	6≡	.	6≡	4≡	.	.
				8 : 3 : 10	8≡	.	.	.	6≡	.	.
d	,,	,,	,,	8 : 5 : 0	5≡	.	.	.	3≡	0	.
				8 : 3 : 10	5≡	.	.	.	5≡	3≡	.
e	B	0·22	37	8 : 5 : 0	6≡	.	6≡	5≡	.	.	.
	B_1	0·03	,,	8 : 5 : 0	6≡	.	6≡	3≡	.	.	.
	B_2	0	,,	8 : 5 : 0	6≡	.	6≡	0	.	.	.
	B	0·22	37	8 : 5 : 0	2=	.	2=	.	.	.	.
				8 : 3 : 10	8≡	.	7≡	.	.	.	.
	B_2	0	,,	8 : 5 : 0	2=	.	0	.	.	.	.
				8 : 3 : 10	8≡	.	7≡	.	.	.	.
g	B_1	0·03	37	8 : 5 : 0	.	5≡	5≡	2=	.	.	.
				8 : 3 : 10	.	.	8≡	.	.	.	.
Distilled water		0	,,	8 : 5 : 0	.	2≡	0	0	.	.	.
				8 : 3 : 10	.	.	5≡	.	.	.	.

Solutions used for the preliminary immersion:

A, 8 : 2 : 0, similar in composition to the calcifying solution but of lower P content. (This solution can itself effect calcification in bones with very exceptionally high second mechanism activity.)

B, basal solution (Table I) containing neither Ca nor P.

B_1 and B_2, similar to B in all respects except bicarbonate content.

In the last experiment (rat *g*) the bone slices in solution B_1 served as controls for those in distilled water.

The results show that when the bone slices were kept at 37° in solution 8:2:0 or in the basal salt solution there was a gradual decrease in the activity of the second mechanism, which became pronounced after 24 hours' immersion. This loss of activity occurred also, though more slowly, at lower temperatures. In salt solutions containing no bicarbonate and in distilled water the inactivation of the second mechanism was more rapid.

These adverse conditions would appear to have had little effect on the phosphatase mechanism over short periods; but calcification in solution 8:3:10 was reduced after prolonged immersion.

These results indicate that in experiments on calcification *in vitro* undue prolongation of the period in the calcifying solutions at 37° can be of little advantage owing to the gradual decrease in the activity of the calcifying mechanisms. Practical experience has, indeed, shown that the maximum calcification obtainable in any given solution at 37° occurs, as a rule, within 18 hours. At room temperature, on the other hand, in solutions 8 : 10 : 0 and 8 : 4 : 10 calcification has been obtained in 43 hours though absent after 19 hours.

Effect of various vitamin and hormone preparations on calcification in vitro.

Experiments were performed to discover whether vitamin D or hormones, which are known to influence the growth and calcification of the skeleton *in vivo*, would have any demonstrable action on the calcification or decalcification of bone slices *in vitro*. The results of these experiments were all negative and will therefore only be summarised briefly.

Vitamin D. (1) Bone slices from a rachitic rat were moistened with 8 : 2 : 0 solution and irradiated in quartz tubes for periods of 15, 30 and 60 minutes before immersion in 8 : 3 : 0, 8 : 5 : 0 and 8 : 3 :10 solutions for 18 hours.

(2) Bone slices from a rachitic rat were immersed for one hour in an olive oil solution of irradiated ergosterol (radiostol) before immersion in the calcifying solutions.

(3) Bone slices were immersed in 20 ml. of various calcifying solutions (with and without ester) in which 0·1, 0·2 or 0·3 ml. of radiostol was emulsified by shaking. A control solution containing 0·2 ml. of olive oil in emulsion was used in addition to the usual controls.

(4) In other experiments calciferol was used instead of radiostol. It was added to the calcifying solutions in amounts equivalent to 400–40,000 antirachitic units per 100 ml. In all the above experiments, untreated slices from the same rat were immersed in similar calcifying solutions (containing no vitamin preparation) to serve as controls. There was no indication of any effect, beneficial or otherwise, of irradiation or of radiostol or calciferol on calcification *in vitro* either in presence or absence of phosphoric ester.

Parathyroid hormone. (1) Bone slices were immersed for 19 hours at 37° in calcifying solutions with and without ester, and containing in addition 10, 20 and 40 units of Eli Lilly parathormone per 100 ml. No increase or inhibition of calcification was apparent; and it was almost impossible to determine by inspection of the stained slices whether there had been any resorption of the previously calcified shaft trabeculae during the experimental period.

(2) Whole bones, heads of bones or half bones of normal young rats were each immersed in 5 ml. of sterile basal salt solution without calcium or phosphate, half the solutions containing 60 units of parathormone per 100 ml. The vessels containing the bones were rotated for 19 hours at 37°. The very slight increase in the inorganic and organic phosphate and the calcium content of the solutions, which occurred during the experiments, was no greater when parathyroid extract was present than when it was not. There was thus no evidence that calcium or phosphate was brought into solution in any form.

Pituitary extract. In two experiments no definite effect on calcification was observed as a result of adding standard posterior lobe extract (equivalent to

0·1 to 15 I.U.) to calcifying solutions with and without phosphoric ester. Short periods of immersion (3 to 6 hours) were chosen in order that any increase in permeability due to the action of the hormone might be more apparent.

Testicular extract. Experiments similar to the above were carried out with testicular extract, for which we are indebted to Drs D. McClean and W. T. J. Morgan. No indication was obtained of more rapid calcification as a result of increased permeability.

Effect on calcification in vitro *of other substances present in the blood.*

The results of two experiments quoted in Table VI show that neither glycine nor urea in concentrations covering the normal physiological range had any significant effect on the activity of the second mechanism. The amounts of new deposit in tibia slices immersed in solutions 8 : 4 : 0 and 8 : 5 : 0 were nearly the same in presence of these substances as in the controls.

Table VI. *The effect of urea and glycine on calcification* in vitro.

17 hours; 37°; p_H 7·4.

	mg. per 100 ml.				mg. per 100 ml.					
					Urea			Glycine		
Rat	Ca	P inorg.	P org.	Control	30	50	60	20	40	50
a	8	4	0	5 =	2 ≡	5 ≡	.	7 ≡	2 =	.
	8	5	0	6 ≡	7 ≡	4 ≡	.	7 ≡	7 ≡	.
b	8	4	0	1 =	.	.	< 1 −	.	.	< 1 −
	8	5	0	5 ≡	.	.	6 ≡	.	.	5 ≡
	8	5	0	4 ≡	.	.	5 ≡	.	.	4 ≡

Table VII. *Effect of sugars and sugar alcohols on calcification* in vitro.

	mg. per 100 ml.				mg. per 100 ml.					
					Glucose		Fructose	Sucrose	Dulcitol	Erythritol
Rat	Ca	P inorg.	P org.	Control	50	100	100	100	100	100
a	10	3	0	5 =		0	.	.	.	.
	10	5	0	7 ≡		0	.	.	.	.
b	10	3	0	1 −			.	.	.	.
	10	4	0	4 =	0	< 1 −	.	.	.	.
	10	5	0	.	4 ≡	0	.	.	.	.
	10	6	0	.	4 ≡	2 =	.	.	.	.
	10	7	0	.	6 ≡	5 ≡	.	.	.	.
c	8	4	0	2 =	.	0	3 −	4 =	.	.
	8	5	0	4 ≡	.	0	2 ≡	2 ≡	.	.
d	8	4	0	4 ≡	.	0	.	.	3 ≡	3 =
	8	5	0	5 ≡	.	< 1 −	.	.	5 ≡	5 ≡

On the other hand the results set out in Table VII show that glucose in concentrations of 50 or 100 mg. per 100 ml. had a very definite inhibitory effect on calcification in inorganic solutions. Thus in rat *b* calcification was less extensive in 10 : 6 : 0 solution containing 100 mg. glucose per 100 ml. than in 10 : 4 : 0 solution containing none. In another paper [Rosenheim, 1934, 1] it is shown that this inhibition is exerted also in presence of protein and is additive to the effect of the latter.

It was at first thought that this inhibition might be explained by the combination of the glucose with the calcium ions; but as will be seen from Table VII

no such marked effect could be demonstrated with other sugars or sugar alcohols. Glycogen also, in concentration of 150 mg. per 100 ml., did not affect the amount of calcification in solution 8 : 5 : 0.

Effect of fluoride, iodoacetate and arsenate on calcification in vitro.

The results given in Tables VIII and IX show that both sodium fluoride and sodium iodoacetate exert a most pronounced inhibitory effect on the second mechanism. The former in concentration as low as 0·0001 *M* completely prevented calcification even in solutions 8 : 6 : 0 and 8 : 6 : 10, though calcification still occurred in solution 8 : 6 : 30. In still lower concentration (0·00001 *M*)

Table VIII. *Effect of sodium fluoride on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control			Concentration of NaF: 0·00001 *M*	0·0001 *M*	
Ca	P inorg.	P org.	*a*	*b*	*c*	*a*	*b*	*c*
8	5	0	5≡	4≡	4=	0	0	0
8	6	0	.	.	.	4≡	0	0
8	3	10	8≡	7≡	5≡	.	0	.
8	5	10	.	.	.	7≡	.	0
8	6	10	.	.	.	.	0	.
8	6	30	.	.	.	.	.	8≡

The results given in columns *a*–*c* were obtained with bone slices from three different rats.

Table IX. *Effect of sodium iodoacetate on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control						Concentration of iodoacetate: 0·0001 *M*	0·001 *M*				
Ca	P inorg.	P org.	*a*	*b*	*c*	*d*	*e*	*f*	*b*	*a*	*b*	*c*	*d*	*e*	*f*
8	5	0	6≡	5≡	4≡	5≡	6≡	5≡	<1−	0	0	0	.	3=	3≡
8	6	0	.	.	.	.	.	.	.	.	.	0	0	.	3≡
8	3	10	6≡	8≡	.	6≡	6≡	.	6≡	0	<1−	.	.	6≡	4=
8	5	10	.	.	9≡	.	.	.	.	.	.	0	.	.	6=
8	6	10	.	.	.	.	.	.	.	.	.	0	2=	.	.
8	6	30	.	.	9≡	.	.	.	.	.	.	5−	7≡	.	.

The results given in columns *a*–*f* were obtained with bone slices from six different rats.

sodium fluoride prevented calcification in 8 : 5 : 0 solution although not in solutions 8 : 6 : 0 and 8 : 5 : 10. The inhibitory effect of sodium iodoacetate, although not apparent in such great dilution, was also very marked. In a concentration of 0·001 *M* it prevented or greatly reduced the calcification in the inorganic solutions, while in presence of ester the amount of deposit was generally reduced.

In separate experiments, summarised in Table X, it was shown that neither fluoride nor iodoacetate in concentrations higher than those used in the above experiments affected the rate of hydrolysis of β-glycerophosphate by a preparation of purified bone-phosphatase.

The presence of 0·001 *M* sodium arsenate in the calcifying solutions 8 : 5 : 0 and 8 : 3 : 10 had neither favourable nor inhibitory effect on calcification *in vitro*.

Table X. *Hydrolysis of β-glycerophosphate by bone-phosphatase in presence of fluoride and iodoacetate.*

		Sodium fluoride		Sodium iodoacetate	
	Control	0·001 *M*	0·01 *M*	0·001 *M*	0·01 *M*
Inorganic phosphate (mg. P) liberated in 1 hour at 37° and p_H 8·6 by 1 mg. phosphatase	0·285	0·275	0·28	0·28	0·275

Each value is the mean of four determinations.

Discussion.

Although the work described in this and the succeeding papers has not yielded the exact knowledge of the second mechanism which we sought, the results have enabled us to extend and to correct our previous information as to the qualities of this mechanism and its relationship to phosphatase, while new ideas have emerged which may usefully form a basis for further experiment. A brief survey may be attempted.

The phosphatase hypothesis in its simplest form assumed that the blood is normally in equilibrium with the bone salt and that a condition of supersaturation, which is the theoretical requirement for precipitation of this salt, is produced locally in hypertrophic cartilage and osteoid by the phosphatase of these tissues acting on phosphoric esters derived from the blood. That calcification could be effected in this way was made clear by experiments *in vitro*; while further evidence of various nature emphasised the intimate connection of phosphatase with bone development.

But it was apparent that this simple scheme is not in itself sufficient. A difficulty arises from the presence of phosphatase in many tissues other than bone. It is true that phosphatases are required for other physiological processes as well as for calcification; it is true also that the phosphatase activity of the bone is high and that it is highest at the period when calcification is most active; but the question remains, if the enzyme in these other tissues sets free inorganic phosphate in a fluid already saturated with the calcium salt, why is this salt not deposited in these tissues as it is in bone? Certain of these tissues are liable to become calcified in abnormal or pathological conditions and the relation of this tendency to the distribution of phosphatase is being investigated [Rosenheim and Robison, 1934; Macfarlane *et al.*, 1934]. We incline to believe that the effect of the enzyme in such cases can, at most, be supplementary to other factors.

Another difficulty is that solutions may be considerably supersaturated with the bone salt and yet remain relatively stable. Unpublished experiments have shown that materials of different types may be immersed in such solutions for several days without any deposit being formed upon them. Clearly, some further qualities, differentiating hypertrophic cartilage and osteoid from other tissues, were required; and the experiments of Shipley *et al.* [1926], fully confirmed in this laboratory, proved that such qualities exist.

In our previous paper [Robison *et al.*, 1930] we showed how the calcifying power attributable to these special qualities of hypertrophic cartilage and that due to the phosphatase mechanism could be separately demonstrated *in vitro* under suitable experimental conditions. An empirical distinction between the two mechanisms was thus established but this did not exclude the possibility of their intimate relationship. For the present it is convenient to discuss them

as separate components of a dual system by which the normal process of calcification is effected in cartilage and bone.

We have evidence that both mechanisms arise in hypertrophic cartilage and osteoid tissue and reach their highest activity with the full histological development of these tissues, thereafter becoming weaker as tissue degeneration proceeds[1] [Fell and Robison, 1929; 1930; Rosenheim 1934, 2]. It has been shown also that the power of the second mechanism varies considerably in the cartilage of different animals of the same species and even of the same litter and that this variation is connected in some way with the condition of the animal; it may possibly be related to its store of vitamin D, although at present the evidence for this is slight and indirect [Rosenheim, 1934, 2].

The second mechanism appears to be more sensitive than the phosphatase to various agents and conditions. It is greatly weakened by potassium cyanide, by treatment of the cartilage with various solvents or by its desiccation. It is not, however, entirely destroyed by these agencies, for even after such treatment the cartilage retains a greater tendency to become calcified in highly supersaturated inorganic solutions than is ever possessed by the aorta or kidney. The activity of the second mechanism also declines more rapidly than that of the phosphatase after the bones are removed from the body and the slices placed in the experimental salt solutions at 37°. In distilled water or in absence of bicarbonate the decline is still more rapid but is retarded in the salt solutions at lower temperatures. These facts suggest that the second mechanism is bound up with some labile structure of the tissue colloids but does not depend for its functioning on the living cell.

Many of the experimental findings could be readily explained by attributing to this mechanism the properties of a trigger, its effect on the supersaturated tissue fluid being similar to that of a crystal of sodium sulphate on the supersaturated solution of this salt. Conceivably, surface forces at fibrillar interfaces might exert some such action, though experiments in this direction have given only negative results.

Another suggestion [Robison and Soames, 1924] that the p_H within the cartilage is raised above that of the surrounding medium, also lacks experimental support.

Some of the results recorded in this paper suggest strongly that the second mechanism is, like the phosphatase, enzymic in nature; while none of the experimental data is inconsistent with this view. In particular the very pronounced inhibitory action of sodium iodoacetate and sodium fluoride recalls the effect of these substances on the enzymes of yeast and muscle, preventing the fermentative breakdown of phosphoric esters in the cycle of changes.

Prior to these experiments with iodoacetate and fluoride Harris [1932] had drawn attention to the parallelism between the presence of phosphatase and the storage of glycogen in cartilage and had put forward the very interesting suggestion that the chemical system in the ossification process might bear some relation to that in yeast and muscle cells. He suggested that the "hypertrophic cartilage cells provide both the phosphatase enzyme and the glycogen; the latter on hydrolysis yields hexosephosphoric esters which, under the action of the phosphatase and the calcium of the circulating body fluids, lead to the deposition of an insoluble phosphate of calcium in the matrix." Fell and Robison [1933] pointed out that phosphatase is also actively secreted by the osteoblasts which, according to Harris, are devoid of glycogen; but they considered that these facts are not necessarily inconsistent with his suggestion.

[1] Other experiments bearing on this point will shortly be published by Fell and Robison.

It may be that the bone is not entirely dependent on the blood for its supply of phosphoric esters, but that these are also synthesised in the hypertrophic cartilage and osteoid as a stage in the building-up and breaking-down of glycogen. A cycle of synthesis and subsequent hydrolysis in the matrix could obviously serve as a mechanism for raising the level of inorganic phosphate at the site of calcification. Phosphatase might thus prove to be an essential component of the second mechanism, the whole consisting of a complex system analogous to that in yeast and muscle. Part of this system might be inactivated by such agencies as acetone or desiccation, or inhibited by potassium cyanide, iodoacetate or fluoride; while the phosphatase, being unaffected, could still function if phosphoric esters were supplied. To these suppositions a parallel might readily be found in the effect of various agents on the fermentative complex of yeast.

It may be noted that while calcification *in vitro* of hypertrophic cartilage can be effected by agency of the phosphatase mechanism when the second mechanism has been inhibited or destroyed, the converse, calcification by second mechanism activity after destruction of phosphatase, has never been demonstrated. Further, it has been shown that tissues such as kidney may possess a high phosphatase activity without the second mechanism, but no tissue has been found which possesses an active second mechanism without phosphatase.

On the whole we may say that the results of our experiments emphasise the possible enzymic nature of the second mechanism and its close relationship with the phosphatase. They justify certain comparisons with the enzyme systems of yeast and muscle and thus lend support to the stimulating suggestion of Harris. The value of this suggestion may not necessarily depend on the proof that glycogen storage precedes calcification—a contention concerning which there appears to be some doubt.

The analogy between the systems of bone and muscle cannot be pressed too far, since muscle does not contain the bone-phosphatase nor does it become calcified. Nor does the more general conception of the second mechanism as an enzyme system in any way preclude the possibility that factors of a different type, such as surface forces in the colloidal matrix, may also assist in promoting the deposition of calcium salts from the tissue fluids after supersaturation has been achieved by enzyme action.

The experiments on the deposition *in vitro* of barium, strontium and magnesium salts show that the second mechanism is not entirely specific for the calcium salt of bone; but they also indicate that the properties of this calcium salt are very specially appropriate to the function which it fulfils. Although magnesium is a normal constituent of bone and teeth, the very high concentration of this element found necessary for its deposition *in vitro* provides evidence that in the body it is precipitated as part of a much less soluble complex salt in which it replaces an equivalent amount of calcium.

The inhibitory influence of proteins and of magnesium and other ions on calcification may probably be explained by their effects on the ionisation of calcium and phosphate, though the possibility of some direct action on the second mechanism is not excluded. It is not so easy to account for the marked inhibitory action of glucose which has also been investigated by zu Hörste [1932]. The reason may perhaps be bound up with the possible relation of the second mechanism to carbohydrate breakdown.

The beneficial effect of bicarbonate on calcification in inorganic solutions was considered in our previous paper to supply some evidence that the deposited bone salt is a complex carbonato-phosphate. The present experiments suggest

an additional or alternative explanation—that the presence of bicarbonate in the calcifying solutions favours survival of second mechanism activity. Bicarbonate may serve to neutralise lactic or other acids produced by the tissue enzymes, thus preventing a rapid fall in the internal p_H of the cartilage. Before accepting this explanation we must know to what extent the internal p_H is independent of that of the external medium, since the bicarbonate-free solutions which failed to produce calcification did not themselves show any significant fall in p_H, and since immersion of the bone slices in solutions of p_H 5·4 for one hour was not found to affect the calcifying power of the cartilage. The question of the internal p_H of the cartilage and osteoid matrix bears so directly on the problems of calcification that some method by which it could be measured immediately before calcification begins and without injury to the calcifying mechanism would be of the greatest value.

The gradual decline in the calcifying power of the bone slices after excision, and particularly in the activity of the second mechanism, points to a limitation of this experimental method of studying calcification *in vitro*. To counterbalance the effect of this loss of power, somewhat higher concentrations of the essential ions than are adequate for progressive calcification in the body may be required for deposition in the restricted period *in vitro*, so that direct comparison of the limiting concentrations *in vitro* with those *in vivo* may be misleading. The method remains, however, most valuable for the comparative study of factors which may influence either the concentrations of these ions or the activity of the mechanisms.

Summary.

1. The nature and properties of the dual calcifying mechanism of hypertrophic cartilage have been further investigated.

2. It was shown that α- and β-glycerophosphates, hexosemonophosphate, fructosediphosphate and trehalosemonophosphate are satisfactory substrates for the bone-phosphatase in calcification *in vitro*. Diphosphoglycerate and lecithin were not effective.

3. Deposits of the salts of barium, strontium and magnesium were obtained in the hypertrophic cartilage *in vitro* by the phosphatase mechanism. Deposits of strontium salts were also obtained from highly supersaturated inorganic solutions containing no phosphoric ester. The deposits were formed in the matrix of the hypertrophic cartilage and usually resembled those of calcium salts. The second mechanism is, therefore, not entirely specific for the calcium salt of bone.

4. Calcification occurred in presence of 0·001 *M* potassium cyanide in bone slices immersed in highly concentrated inorganic solutions when the original second mechanism activity of the bone was high. The inhibition of the second mechanism by potassium cyanide previously reported is, therefore, not complete.

5. The two components of the calcifying mechanism could not be inactivated differentially by heating the bone slices at different temperatures or by soaking them in isotonic salt solutions at different p_H levels.

6. A gradual decline in the activity of both mechanisms occurred when the excised bone slices were immersed in various salt solutions at 37°. This decline was more rapid for the second mechanism than for the phosphatase. It was more rapid also in absence of bicarbonate or in distilled water, but was retarded at lower temperatures.

7. Vitamin D or hormone preparations known to influence the growth and calcification of the skeleton *in vivo* had no demonstrable action on the calcification or decalcification of bone slices *in vitro*.

8. Glucose in concentrations of 50–100 mg. per 100 ml. had a very definite inhibitory effect on calcification *in vitro* in inorganic solutions, but no such effect could be demonstrated for fructose, sucrose, dulcitol or erythritol. Glycine and urea, also, in concentrations covering the normal physiological range were without significant effect on the activity of the second mechanism.

9. Sodium fluoride in concentrations as low as 0·00001 *M* and sodium iodoacetate in concentration of 0·0001 *M* exerted a pronounced inhibitory effect on the second mechanism, but not on the phosphatase.

10. The nature of the dual calcifying mechanism is discussed in the light of these and other experimental results.

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DESCRIPTION OF FIGURES IN PLATE I.

Figs. 1 to 4. Deposits of magnesium and strontium salts in the hypertrophic cartilage zone stained with silver nitrate.

Figs. 5 and 6. Crystalline deposit of barium salt unstained.
X = deposit of Mg, Sr or Ba salt formed *in vitro*.

Fig. 1. Microtome section of a tibia slice after immersion for 17 hours in solution 0·1 *M* Mg : 10 : 60 containing high concentrations of magnesium and phosphoric ester. Note the extensive deposit of magnesium salt in the matrix of the previously uncalcified hypertrophic cartilage zone. Magnification ×20.

Fig. 2. Tibia slice after immersion for 19 hours in the inorganic solution 0·01 *M* Sr : 12 : 0. Note the dense deposit of strontium salt formed in the hypertrophic cartilage zone in absence of phosphoric ester. Magnification ×8.

Fig. 3. Microtome section of part of a tibia slice after 19 hours in the solution 0·005 *M* Sr : 8 : 30, containing phosphoric ester. Magnification ×25.

Fig. 4. Deposit of strontium salt formed in 19 hours in solution 0·005 *M* Sr : 0 : 30. The appearance of the deposit in the matrix of the hypertrophic cartilage cells closely resembles that of a normal deposit of calcium salt. Magnification ×160.

Fig. 5. Barium salt deposited in crystalline form in the hypertrophic cartilage zone of the tibia of a rachitic rat immersed for 19 hours in solution 0·005 *M* Ba : 5 : 30. Magnification ×25.

Fig. 6. The same crystals photographed by polarised light between crossed Nicols. Magnification ×50.

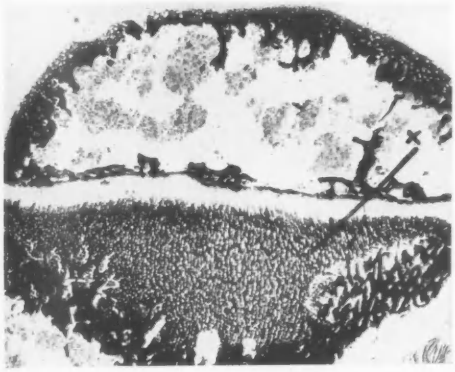

Fig. 1.

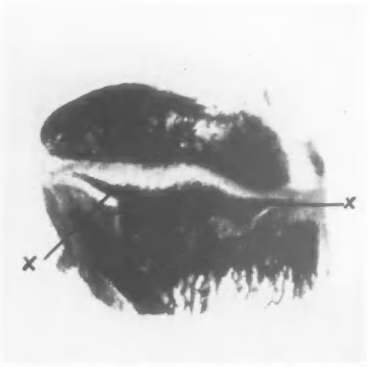

Fig. 2.

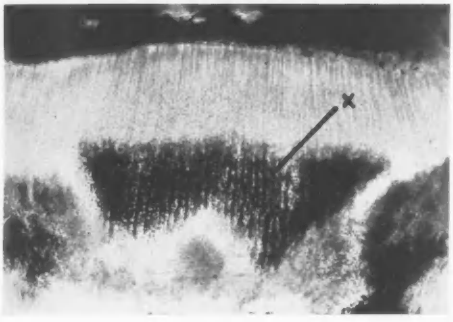

Fig. 3.

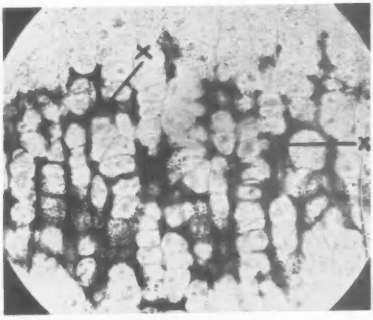

Fig. 4.

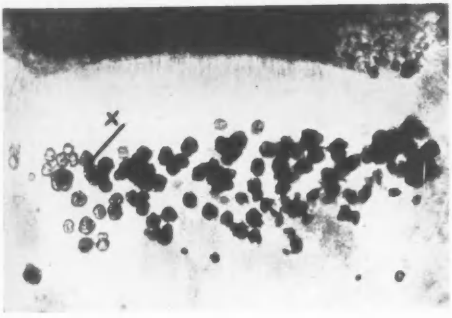

Fig. 5.

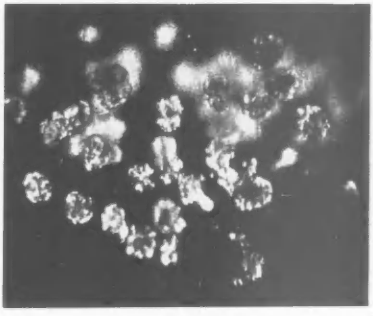

Fig. 6.

XCVI. THE EFFECT OF SERUM-PROTEINS ON CALCIFICATION *IN VITRO*.

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The inhibitory effect of egg-albumin on the calcification of bone slices in organic salt solutions was noted by Shipley *et al.* [1926]. The concentrations of calcium and inorganic phosphate in the solutions used by these workers were approximately those found in normal serum; but as was pointed out by Robison [1926], this apparent similarity is misleading owing to the absence of the other constituents of plasma, particularly the proteins, which are present in a concentration of 7 % and greatly reduce the ionisation of calcium. In Shipley's solutions as little as 2 % egg-albumin was sufficient to inhibit calcification. To bring about deposition of the bone salt from plasma, therefore, some mechanism for increasing the concentration of calcium or phosphate ions would be required, and such a mechanism is furnished by the bone-phosphatase.

The experiments described in this paper were planned to study the effect of serum-proteins on the calcification of hypertrophic cartilage *in vitro*, both in inorganic solutions and in those containing phosphoric ester.

Experimental.

The method of experiment was similar to that described by Robison *et al.* [1930]. Slices from the heads of the long bones of rachitic rats were immersed for periods of 16 to 20 hours in the sterile experimental solutions at 37° and p_H 7·4. They were then fixed, stained with silver nitrate and cleared in cedarwood oil. The amount of new calcification in the zone of hypertrophic cartilage was judged by examination under low magnification and is shown in the tables by a figure (maximum 10) representing the extent of the deposit, and bars (maximum 5) indicating the density, thus $6\equiv$. Except where otherwise stated each experiment was carried out with bone slices from the same rachitic rat.

Only those modifications in the technique necessitated by addition of protein will be described in greater detail.

Serum-proteins were obtained from normal horse serum by saturation with ammonium sulphate, followed by solution of the precipitate and reprecipitation. The ammonium sulphate precipitate was dialysed in cellophane sacs against hot tap water, distilled water and finally 0·8 % NaCl, and the stock protein solution thus obtained was filtered through Seitz filters and stored at 0° in a sterile bottle fitted with a syphon for withdrawal of samples. The protein content of

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the solution was estimated by Kjeldahl nitrogen determinations and also by refractometer[1].

The stock protein solution used for most of the experiments quoted contained 0·76 g. N per 100 ml. (≡ 11 % protein), and further, 0·8 g. NaCl, 9·55 mg. Ca, 0·53 mg. Mg and 0·1 mg. P per 100 ml. This was diluted as required with a basal salt solution of such concentration that the final salt contents of the calcifying solutions containing protein and of the protein-free solutions should be the same.

Protein-salt solutions containing the requisite amounts of calcium and inorganic and organic phosphate, and made up in slightly more concentrated form than finally required, were sterilised by filtration through a Seitz filter into a pyrex flask[2].

From the sterile protein-salt solutions the final solutions were prepared in 20 ml. flasks by addition of water, glucose or other constituents as required. The p_H of these solutions was adjusted to 7·35–7·4. Measurements were made with a capillator with correction for protein error and were checked by occasional electrometric measurement.

The sterility of the solution in each flask was tested at the end of the experimental period by incubating a drop on an agar slope. Experiments in which there was serious contamination with consequent fall in p_H were rejected.

The inhibitory effect on calcification in vitro *of protein in various concentrations.*

In the experiment quoted in Table I, bone slices from the same rat were immersed in solutions containing 0, 1·5 and 3 % serum-proteins at various

Table I. *Effect of serum-proteins on calcification* in vitro.

16 hours; 37°; p_H 7·4.

mg. per 100 ml.			Protein g. per 100 ml.		
Ca	P inorg.	P org.	0	1·5	3
			Calcification *in vitro*		
10	4	0	6 ≡	2 =	0
10	6	0	.	6 ≡	7 =
10	4	3	8 ≡	8 ≡	.
10	4	10	.	8 ≡	7 ≡

All the bone slices were from the same rachitic rat.

levels of calcium, inorganic and organic phosphate. In absence of protein there was good calcification in the inorganic solution; 10:4:0[3] but calcification in

[1] For assistance in preparing these solutions, and for the refractometer measurements I am indebted to Dr W. T. J. Morgan.

[2] Estimations of calcium and magnesium were at first made on the non-sterile solutions before filtration, but later it was found that appreciable amounts of Mg and Ca were being extracted from the Seitz asbestos pads. In this way the concentrations of Mg and Ca were being raised by as much as 0·7 mg. Mg and 1 to 1·5 mg. Ca per 100 ml.

Tests showed (*a*) that silica was not extracted simultaneously, and (*b*) that all the soluble Mg and Ca could be extracted by first passing through the pads 50 ml. of a solution similar to the basal salt solution but free from Ca and Mg. This modification of the technique described above was adopted in all further experiments, although the certainty of sterilisation by filtration was thereby somewhat lessened. Only a few of the early experiments in which the Ca and Mg concentrations were raised by filtration have been quoted, and these are marked with an asterisk.

[3] The experimental solutions are referred to in this way throughout the text. Thus, solution 10 : 4 : 3 contains 10 mg. Ca, 4 mg. P as inorganic phosphate and 3 mg. P as phosphoric ester per 100 ml.

this solution was partially inhibited by 3 % protein. In solution 10 : 6 : 0 and in the ester solution 10:4:10 calcification still occurred even in presence of 3 % protein.

Table II. *Effect of serum-proteins on calcification* in vitro.

17 hours; 37°; p_H 7·4.

mg. per 100 ml.			Rat	*a*		*b*		*c*		*d*		*e*	
Ca	P inorg.	P org.	Protein g./100 ml.	0	7	0	7	0	7	0	7	0	7
10	3	0		.	.	1−	.	.	.	.	.	0	.
10	4	0		6≡	.	2=	0	7≡	0	2≡	.	2=	0
10	5	0		.	.	6≡	0	.	2=	.	0	.	0
10	6	0		.	1=	.	4≡	.	5≡	.	0	.	0
10	7	0		.	2=	.	5≡	.	7≡	.	0	.	.
10	8	0		.	5≡	.	.	.	.	.	2≡	.	.

The concentration of protein was raised to 7 % in the experiments quoted in Table II. Considering first the ester-free solutions, it will be seen that the lowest level of Ca × P at which calcification regularly took place was 10 × 6 in presence of protein as compared with 10 × 3 in absence of protein. In one experiment only, out of a large number, was calcification obtained at the lower level 10 × 5 in protein solution (rat *c*). Where the calcifying power of the bones was low, as in rat *d*, a level as high as 10 × 8 was necessary in the protein solutions before calcification occurred.

The inhibitory effect of glucose on calcification in protein-salt solutions.

It is shown elsewhere that glucose has an inhibitory effect on calcification *in vitro* in protein-free solutions [Robison and Rosenheim, 1934]. From the results given in Table III it will be seen that this inhibitory effect was equally marked in 7 % protein solution. Inhibition due to 0·01 % glucose was not demonstrable, but in presence of 0·03 % there was total inhibition of calcification in solution 10 : 7 : 0 and partial inhibition in solution 10 : 9 : 0. In presence of 0·05 or 0·1 % glucose calcification was only twice obtained at a level lower than 11 × 10 in a total of 11 experiments, but no obvious progressive inhibition of calcification was observed with concentrations of glucose greater than 0·1 %.

Table III. *Effect of glucose on calcification* in vitro *in presence of protein.*

Protein 7 g./100 ml.; 18 hours; 37°; p_H 7·4.

	mg. per 100 ml.			Glucose mg. per 100 ml.						
Exp.	Ca	P inorg.	P org.	0	10	30	50	75	100	150
34	10	6	0	4≡	.	.	.	.	0	.
	10	7	0	5≡	.	.	.	.	1=	.
30	10	8	0	2≡	.	.	.	.	0	.
	10	10	0	.	.	.	.	.	0	.
17*	10	7	0	4=	4=	0	0	.	0	.
	10	9	0	7≡	7≡	3≡	0	.	0	.
15*	11	9	0	7≡	.	.	0	.	0	.
	11	10	0	9≡	.	.	4=	.	5≡	.
23*	11	10	0	9≡	.	.	5≡	3≡	2=	2=
	10	12	0	9≡	.	.	.	5≡	3=	6≡

The inhibitory effect of magnesium on calcification in protein-salt solutions.

It has been shown [Shipley and Holt, 1927; Kramer *et al.*, 1927; Robison *et al.*, 1930] that magnesium exerts a definitely inhibitory effect on calcification in inorganic solutions, so that to obtain a given amount of deposit progressively higher levels of the Ca × P product are needed as the magnesium concentration is increased. The two experiments given in Table IV show that the same effect is exerted also in presence of glucose and protein.

Table IV. *Effect of magnesium on calcification* in vitro *in presence of protein and glucose.*

Protein 7 g./100 ml.; glucose 100 mg./100 ml.; 17 hours; 37°; p_H 7·4.

Rat *a*					Rat *b*				
mg. per 100 ml.			mg. Mg per 100 ml.		mg. per 100 ml.			mg. Mg per 100 ml.	
Ca	P inorg.	P org.	1·1	3·2	Ca	P inorg.	P org.	2·6	4·1
11	7	0	2 =	0	10	6	0	0	.
11	8	0	1 =	0	10	7	0	2 =	.
11	9	0	1 =	0	10	8	0	3 =	0
11	10	0	4 ≡	2 =	10	9	0	6 ≡	3 =
11	12	0	7 ≡	6 ≡	10	10	0	.	5 =
					10	12	0	.	8 ≡

It will be seen that with a low magnesium concentration of 1·1 mg. per 100 ml. calcification was obtained in bone slices from rat *a* at the Ca × P level 11 × 7, in presence of 7 % protein and 0·1 % glucose. When the magnesium concentration was increased to 3·2 mg. per 100 ml. no calcification was obtained at a level lower than 11 × 10.

In bone slices from rat *b* calcification was obtained in presence of 7 % protein, 0·1 % glucose and 2·6 mg. Mg per 100 ml. at the exceptionally low Ca × P level of 10 × 7 and in presence of 4·1 mg. Mg, at the level 10 × 9. The inhibition due to increased magnesium concentration is here still apparent, although the bones used for this experiment showed a definitely higher calcifying potentiality than usual, thus further illustrating that variability discussed elsewhere [Rosenheim, 1934].

The beneficial effect of phosphoric ester on calcification in protein-salt solutions.

The effect of sodium glycerophosphate on calcification in presence of 7 % protein was next investigated. Results obtained in glucose-free solutions are given in Table V. They show that there was no calcification at levels lower than 12 × 6 in absence of ester, whereas in presence of glycerophosphate equivalent to 3 mg. P per 100 ml. calcification occurred at the level 12 × 4. In solution 12 : 4 : 10 calcification was more extensive and denser than in solution 12 : 7 : 0.

The results given in Table VI show that the beneficial effect of phosphoric ester was equally marked in solutions containing 0·1 % glucose.

Table V†. *Effect of phosphoric ester on calcification* in vitro *in presence of protein.*

Protein 7 g./100 ml.; 17 hours; 37°; p_H 7·45.

	mg. per 100 ml.		Glycerophosphoric ester mg. organic P per 100 ml.		
Exp.	Ca	P inorg.	0	3	10
5*	12	4	0	0	6≡
	12	6	2−	.	.
7*	12	4	.	4=	9≡
	12	5	0	5≡	7≡
	12	6	3=	.	.
	12	7	6≡	.	.

† It will be noted that the slightly increased Ca and Mg concentrations in the solutions used for the experiments quoted in this and the following table (see footnote 2, p. 700) do not in any way affect the conclusions drawn, as the results in each experiment are on a strictly comparable basis.

Table VI. *Effect of phosphoric ester on calcification* in vitro *in presence of protein and glucose.*

Protein 7 g./100 ml.; glucose 100 mg./100 ml.; 37°; p_H 7·4.

		mg. per 100 ml.		Glycerophosphoric ester mg. organic P per 100 ml.				
Exp.	Time (hours)	Ca	P inorg.	0	0·5	1	3	10
8*	17	12	4	.	.	.	0	3≡
		12	5	0	.	0	0	.
		12	6	0	.	.	.	.
		12	7	2≡	.	.	.	.
9*	17	12	5	.	.	.	0	7≡
		12	6	0	0	0	0	7≡
11*	17	12	6	0	.	.	3≡	5≡
		12	7	0	.	0	2≡	6≡
		12	8	0	.	1=	4≡	.
12*	10	12	8	0	.	3−	4=	8≡
		12	9	4=	4=	7−	7=	8=
14*	17	10	8	0	.	0	5≡	.
		10	9	0	.	0	6≡	.
		10	10	5≡	.	6≡	7≡	.

Calcification in vitro *in the serum of various species.*

Having obtained the above data with regard to calcification *in vitro* in artificial protein-salt solutions, a further series of experiments was carried out with the sera of normal animals.

It was essential to obtain the blood without the use of anaesthetics, at the same time avoiding any condition involving fright or surgical shock, all of which may lead to a rapid increase in the inorganic phosphate of the serum. It was equally important to avoid haemolysis even in slight degree, since this would liberate appreciable amounts of phosphoric ester from the red blood corpuscles.

Serum was obtained from adult rabbits by bleeding from the ear vein and from young rabbits and rats by heart puncture, precautions being taken to maintain sterility. Some difficulty was encountered in obtaining sufficient sterile serum from young rats for the calcification experiment and for the necessary analyses.

The serum was separated from the corpuscles as rapidly as possible to avoid the alteration in inorganic phosphate content which may occur when whole blood stands at room temperature [Martland and Robison, 1926]. The serum was then measured into small sterile tubes, and when required, inorganic phosphate was added in the most concentrated form possible, the dilution of the serum not usually exceeding 0·5 %. The p_H was adjusted to 7·4 with sterile alveolar air or CO_2. In all the experiments quoted, control slices from the same animal were immersed for the same length of time in solution 8:4:0 or 8:5:0 and good calcification was invariably obtained even when the volume of solution was reduced to 2 ml. In small volumes of serum the p_H tended to fall slightly during the experimental period.

In Table VII are shown the results of experiments on the calcification of bone slices of rachitic rats in the serum of normal rats. They are arranged in order of increasing Ca × P product of the serum. Results (1)–(4) in any one experiment were obtained with bone slices from the same animal. The negative result obtained with the serum of one rachitic rat (*R*), in which the Ca × P product was only 28, is included in this table.

The lowest level of the Ca × P product at which there was calcification without addition of extra phosphate was 87 (Exp. 15 (1) and (2)). No calcification occurred in 17 hours in a normal serum of Ca × P level 57 (Exp. 13 (1)), but in samples of the same serum to which inorganic phosphate was added to bring up the levels to 68 and 80 respectively there was slight calcification.

Table VII. *Calcification* in vitro *in rat serum.*

			mg. per 100 ml.				
				Inorganic P			
Exp.	Vol. of serum ml.	Time (hours)	Ca	Serum	Added	Ca × P	Calcification
*R*16	2·5	17	10·3	2·7	.	28	0
13 (1)	2·0	17	9·1	6·3	.	57	0
(2)	,,	,,	,,	,,	1·2	68	<1 −
(3)	,,	,,	,,	,,	2·5	80	3 =
15 (1)	,,	16	9·9	8·8	.	87	3 =
(2)	,,	40	,,	,,	.	,,	3 =
10	2·5	17	9·5	9·6	.	91	8 ≡
9	3·0	16	11·2	8·2	.	92	6 =
7	2·0	18	12·1	8·6	.	104	8 ≣
Y 6	2·0	17	10·9	11·7	.	129	7 ≡

R = Rachitic young rat. *Y* = Normal young rat.

Experiments on the calcification of rat bones in rabbit serum are summarised in Table VIII. The serum-calcium of rabbits is higher than that of rats and subject to considerable fluctuations [Culhane, 1927; Kapsinow and Underhill, 1929] but in the samples used the product Ca × P was generally lower than in rat's serum. A Ca × P level higher than 77 could not be reached without addition of inorganic phosphate, and calcification was not obtained in adult rabbit's serum without such addition, the lowest level for calcification being 87, artificially produced. The inorganic phosphate in the serum of young rabbits is, however, slightly higher than in that of the full-grown animals, and in one such serum with a Ca × P product of 109, slight calcification occurred in 41 hours.

Table VIII. *Calcification* in vitro *in rabbit serum.*

Exp.	Vol. of serum ml.	Time (hours)	mg. per 100 ml. Ca	Inorganic P Serum	Inorganic P Added	Ca × P	Calcification
3 (1)	5	40	15·8	3·0	.	47	0
11 (1)	2	17	17·5	2·84	.	50	0
(2)	,,	,,	,,	,,	.	,,	0
8 (1)	1	18	14·4	3·8	.	55	0
(2)	2	,,	14·0	4·0	.	56	0
2 (1)	5	40	14·0	4·0	.	56	0
(2)	,,	,,	,,	,,	1·0	70	0
1 (1)	6	16	15·0	5·12	.	77	0
(2)	,,	40	,,	,,	.	,,	0
3 (2)	5	19	15·8	3·0	2·5	87	1 −
2 (3)	,,	40	14·0	4·0	2·3	88	2 =
8 (3)	2	18	14·0	4·0	2·3	,,	3 =
11 (3)	,,	17	17·5	2·8	2·5	93	5 ≡
(4)	,,	,,	,,	,,	3·5	110	5 ≡
3 (3)	5	19	15·8	3·0	2·5	130	8 ≡
(4)	,,	,,	,,	,,	8·0	174	10 ≡
Y 4 (1)	2	23	12·5	8·6	.	108	0
(2)	5	,,	,,	,,	.	,,	0
Y 5 (1)	,,	41	13·0	8·4	.	109	1 −
(2)	,,	,,	,,	,,	1·8	134	4 ≡

Y = Young rabbit.

Although samples of rat serum containing 10 mg. Ca and 9 mg. P per 100 ml., and rabbit serum containing 15 mg. Ca and 6 mg. P per 100 ml. have the same Ca × P product of 90 there is no suggestion that the same percentage of calcium is ionised in the serum of these two species or that their calcifying power is the same. The assumption that the calcifying power is directly proportional to the simple product Ca × P is theoretically unjustifiable, so that clearly only a limited significance can be attached to a comparison of solutions and sera on this basis.

The fact that calcification in rat serum is not markedly better than in rabbit serum, and only slightly better than in artificial 7 % horse serum-protein solution, disposes of the objection that in some experiments failure to use homologous serum, or rat serum-proteins, might be affecting the results.

Table IX. *Calcification* in vitro *in human serum.*

Exp.	Serum	Vol. of serum ml.	Time (hours)	mg. per 100 ml. Ca	Inorganic P Serum	Inorganic P Added	Ca × P	Calcification
12	Adult	1	17	9·6	3·9	.	37	0
		2	,,	,,	,,	.	,,	0
		,,	,,	,,	,,	1·1	48	0
		,,	,,	,,	,,	2·3	59	5 =
		,,	,,	,,	,,	3·4	69	5 =
		,,	,,	,,	,,	4·6	81	5 ≡
14	Child 10 years	2	18	10·8	5·3	.	57	0
		,,	,,	,,	,,	0·9	66	1 =
		,,	,,	,,	,,	1·8	76	3 ≡
	Child 4 years	,,	,,	10·1	6·3	.	64	1 =

The results of experiments with the serum of human adults and children are given in Table IX. The levels adequate for calcification were lower than in rat and rabbit serum. Definite calcification occurred when inorganic phosphate was added to raise the Ca × P levels from 37 to 59 and from 57 to 66 respectively.

Calcification occurred without added phosphate in serum from a child of 4 years, the Ca × P product being 64[1].

In general it will be seen that the higher the Ca × P product of the solution bathing the bone slices, the denser and more extensive was the new deposit obtained.

Discussion.

It is known that a considerable proportion of the total calcium of blood is present in non-diffusible form and is probably largely combined with the plasma-proteins. The consequent reduction in the concentration of calcium ions must profoundly affect the calcifying power of the plasma, a conclusion which is fully borne out by the results of the experiments described in this paper. The addition of serum-proteins to the inorganic calcifying solutions rendered very much higher levels of the product Ca × P necessary, in order that calcification of the bone slices should take place *in vitro*.

Since calcification in the body may take place more slowly and under more favourable conditions than in these experiments it may reasonably be expected that somewhat lower levels of the product Ca × P than those here required may suffice for calcification *in vivo*. These experiments, therefore, do not give precise information as to the lowest value of this product which would be effective in the body. They show that under the experimental conditions the sera of very young animals, and sometimes also of older animals, may bring about calcification within 20 hours in the hypertrophic cartilage of rachitic rats, but that with many sera it is necessary to increase the phosphate concentration artificially in order that this result may follow.

Still higher levels of the product Ca × P were required with artificial solutions similar to plasma in their content of inorganic salts, protein and glucose, but here, as in all previous experiments, the necessary Ca × P level was notably reduced if phosphoric ester was added.

The better results obtained with natural as compared with artificial sera may well have been due to the small amount of phosphoric ester present in the former; but other constituents of serum not included in the artificial solutions may also have influenced the results.

Summary.

1. The effect of serum-proteins in different concentrations on the calcification *in vitro* of hypertrophic cartilage has been investigated.

2. In presence of horse serum-proteins in 7 % concentration the lowest Ca × P level at which calcification regularly took place in solutions containing no phosphoric ester was 10 × 6 as compared with 10 × 3 in absence of protein.

3. The inhibitory effect of glucose on calcification observed in non-protein solutions was apparent also in presence of 7 % protein and was additional to the effect of the latter, so that in salt solutions containing 0·1 % glucose and 7 % protein calcification was not usually obtained at a level lower than 11 × 10.

4. The inhibitory effect of magnesium on calcification previously observed in non-protein solutions was apparent also in presence of protein.

[1] Shipley *et al.* [1926] have stated that they obtained calcification in bone slices immersed in children's serum of Ca × P product 50 to 54.

5. In presence of glycerophosphoric ester calcification in protein-salt solutions took place at lower levels of the Ca × P product than in its absence. Thus in bone slices from the same rat calcification was obtained in solution 12 : 4 : 3, but not in solution 12 : 6 : 0.

6. Calcification *in vitro* was obtained in the serum of adult rats and rabbits at a Ca × P level of 80–90, and in human serum at a level of about 60. In the serum as drawn with precautions to avoid alteration in inorganic phosphate concentration the requisite level of the Ca × P product was rarely reached, so that the addition of inorganic phosphate was necessary in order that calcification should take place under the experimental conditions.

It is a pleasure to acknowledge my indebtedness to Prof. R. Robison and to thank him for his help and encouragement throughout the course of this work.

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XCVII. VARIABILITY IN THE ACTIVITY OF THE CALCIFYING MECHANISM IN THE BONES OF RACHITIC RATS.

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(*Received February 26th, 1934.*)

It was noted by Robison *et al.* [1930] that the calcifying power attributed to the second mechanism in the hypertrophic cartilage varied considerably in different rachitic rats, even in those of the same age and litter. The variation was manifested by differences in the minimum level of the Ca × P product required for calcification *in vitro* in inorganic solutions and also in the extent of the deposit obtained at higher levels.

Experiments bearing on the relation of such variation to the rate of growth and the condition of the rachitic animals are presented in this paper.

For the first group of experiments litters were selected in which the rats showed unusually varied growth over a 3 to 4-week period on McCollum 3143 diet, and the possible connection of this variation in growth rate with the calcifying power of the cartilage was investigated. The litter-mates were killed on the same day, and tibia slices from each rat were immersed in 20 ml. of the specified calcifying solutions, one slice per flask. The flasks containing the slices were kept at 37° for 20 hours and rotated continuously. The p_H in all the flasks remained constant at 7·4 throughout each experiment.

The growth curves of the rats are given in Fig. 1 and a summary of the experiments in Table I. In the marks assigned to the calcification the figures, with maximum 10, show the approximate extent of the new deposits, and the bars, with maximum 5, indicate the density. The depth of the uncalcified zone in the control slices varied widely, and the figures show the proportion of this zone which became calcified and not the absolute amount of deposit.

The results show that the second mechanism activity developed in the hypertrophic cartilage for the most part varied directly with the amount of growth of the rat during the period on rachitogenic diet.

The figures given for the inorganic solutions (Exps. 1–5) show the variation in the amount of calcification due to the second calcifying mechanism alone. Those given for the ester solutions (Exp. 2) illustrate in an equally striking manner the variation in the calcification produced by the combined mechanisms under conditions which probably approached more closely to those existing *in vivo*.

In inorganic solution calcification at the low level[2] 8 : 2 : 0 was obtained in the case of two of the rats showing the most growth over the period on diet, while in the solutions 8 : 3 : 0 and 8 : 4 : 0 calcification was more general but

[1] Grocers' Company Research Student. The work described in this paper formed part of a thesis approved for the degree of Ph.D. in the University of London.

[2] The experimental solutions are referred to in this way throughout the text. Thus solution 8 : 5 : 3 contains 8 mg. Ca, 5 mg. P as inorganic phosphate and 3 mg. P as organic phosphate per 100 ml. basal salt solution.

decreased in amount with decreasing rate of growth. In solution 8 : 5 : 0 differences were less pronounced.

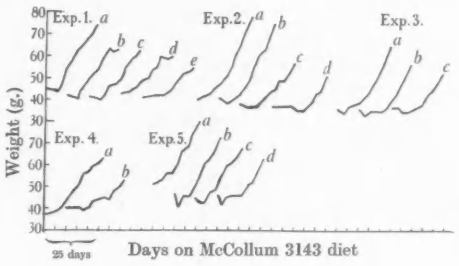

Fig. 1.

Table I.

Exp.	Period on diet (days)	Litter	Rat	Wt. at 21 days* g.	Increase in wt. on diet g.	mg. per 100 ml.	Inorganic solutions				Ester solutions		
						Ca	8	8	8	8	8	8	8
						P inorg.	2	3	4	5	2	2	2
						P org.	0	0	0	0	1	2	3
1	23	2384	*a* ♂	45	29		0	2=	7=	7≡	.	.	.
			b ♀	42	21		0	<1–	4≡	5≡	.	.	.
			c ♀	41	21		0	<1–	4≡	5≡	.	.	.
			d ♀	43	17		0	0	3≡	5≡	.	.	.
			e ♀	41	14		0	0	2≡	5≡	.	.	.
2	25	2661	*a* ♂	40	38		2=	7≡	7≡	.	5≡	7≡	8≡
			b ♂	41	34		0	4=	5≡	.	3=	5≡	7≡
			c ♀	38	19		0	3=	6≡	.	1–	4≡	6≡
			d ♂	37	14		0	1=	4≡	.	0	3=	3≡
3	21	2869	*a* ♀	36	29		1=	7≡	8≡	.	.	.	.
			b ♀	36	21		0	6≡	8≡	.	.	.	.
			c ♀	37	16		0	1=	5≡	.	.	.	.
4	27	2720	*a* ♀	37	26		0	0	1=	5≡	.	.	.
			b ♀	40	13		0	0	0	5≡	.	.	.
5	21	2675	*a* ♂	52	29		0	3≡	4≡	.	.	.	.
			b ♀	47	26		0	4≡	5≡	.	.	.	.
			c ♂	45	23		0	3≡	5≡	.	.	.	.
			d ♂	46	17		0	3=	5≡	.	.	.	.

* All the litters were 21 days old when placed on the rachitogenic diet.

The relation of calcification to growth rate is not apparent in the case of Litter 2675, Exp. 5. It may be noted that these rats were exceptionally well grown at the beginning of the dietary period.

As regularly observed in previous experiments, the amount of new calcification in the slices from any one rat showed a marked gradation in passing from lower to higher levels of the Ca × P product. The use of such material, that is bone slices taken from the same animal, for observing the effect of various factors on the calcifying mechanisms is thus justified.

The favourable effect on calcification of phosphoric ester even in low concentration is again emphasised by the results of Exp. 2.

In another series of experiments quoted in Table II, three or four rats selected from different litters were killed on the same day after different periods of 1 to 6 weeks on McCollum 3143 diet.

Table II.

Exp.	Rat	Days on diet	mg. per 100 ml.		Inorganic solutions				Ester solutions		
			Ca		8	8	8	8	8	8	8
			P inorg.		3	4	5	6	3	4	3
			P org.		0	0	0	0	3	3	10
1	1 ♀	7			.	10 ≡	.	.	.	.	10 ≡
	2 ♀	10			.	10 ≡	.	.	.	.	10 ≡
	3 ♀	28			.	<1 –	.	.	.	.	7 ≡
	4 ♀	37			.	1 =	.	.	.	.	2 ≡
2	1 ♂	7			.	10 ≡	10 ≡	10 ≡	10 ≡	10 ≡	.
	2 ♀	17			.	2 =	3 ≡	3 ≡	4 ≡	5 ≡	.
	3 ♀	30			.	0	<1 =	<1 =	4 ≡	5 ≡	.
3	1 ♂	15			.	3 =	6 =	.	5 ≡	.	10 ≡
	2 ♂	17			.	2 =	6 ≡	.	6 ≡	.	8 ≡
	3 ♂	28			.	<1 –	<1 =	.	6 ≡	.	6 ≡
	4 ♀	40			.	<1 ≡	<1 ≡	.	4 ≡	.	4 ≡
4	1 ♂	28			2 =	7 ≡	8 ≡	.	.	.	10 ≡
	2 ♂	42			<1 –	3 ≡	7 ≡	.	.	.	8 ≡
	3 ♀	56			<1 –	<1 –	6 ≡	.	.	.	7 ≡

The results show that the calcifying power of the cartilage decreases with increasing periods on a rachitogenic diet.

The position of the deposit in these bone slices is a point of considerable interest. In the bones of the young rats which had been less than 2 weeks on the diet (Exp. 1, rats 1 and 2; Exp. 2, rat 1) the whole of the narrow zone of hypertrophic cartilage was apparently in the optimum condition for calcification. Deposition *in vitro* extended from the "line" to the shaft, and the slices calcified *in vitro* resembled bone slices from normal animals of the same age (Plate II, Figs. 2 and 3). In bone slices from the rats which had grown well for 3–4 weeks on a rachitogenic diet, deposition *in vitro* had the appearance familiar in the "line test," that of a band of calcification separated from the diaphysis by a zone of disorganised and less readily calcifiable tissue.

This band of cartilage calcifiable in inorganic solution became narrower as the period on diet increased, until after 4 weeks or more there were large areas of uneroded hypertrophic cartilage which did not become calcified, even in solution 8 : 6 : 0.

It would seem that the calcifying power of the second mechanism is greatest where the cells have just attained full hypertrophy, decreasing after this stage has been passed. A similar conclusion was reached by Niven and Robison in experiments with the cartilage of embryonic rabbit femora [quoted by Robison, 1932]. The same general statement may also be made for the combined calcifying power of the phosphatase and second mechanisms, although the area of cartilage calcified *in vitro* was invariably more extensive in presence of ester than in the corresponding inorganic solutions.

The formation of a calcified line in healing rickets is probably due to the maximum calcifying power of the dual mechanism in this region.

The validity of the argument based on the results shown in Tables I and II may be criticised on the grounds that the observed variations in calcifying power do not in every case run parallel either with the growth rate or with the length of time on the rachitogenic diet. These results do, however, furnish strong evidence that the calcifying power of the cartilage in a rachitic rat varies with the condition of the animal; and this conclusion is supported by the general results of experiments carried out in this laboratory over many years.

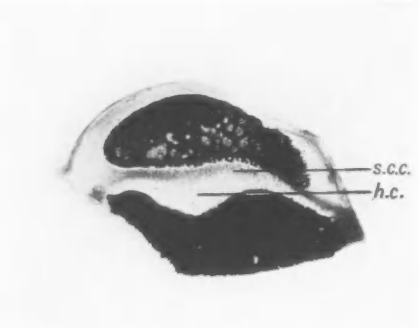

Fig. 1.

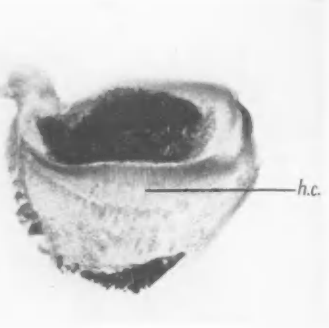

Fig. 4.

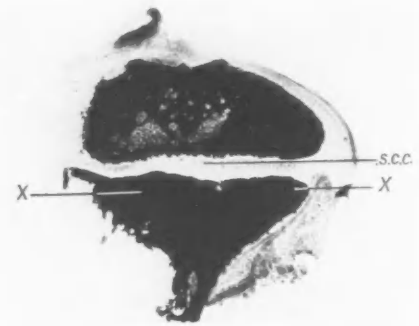

Fig. 2.

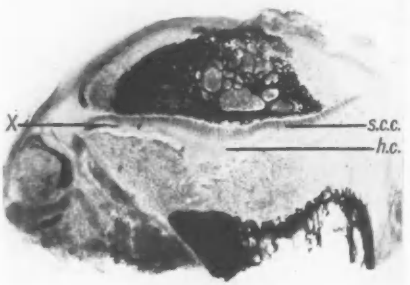

Fig. 5.

Fig. 3.

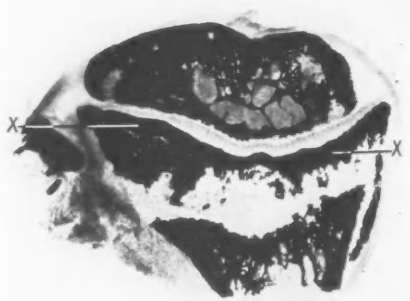

Fig. 6.

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Whether this variation is connected with deficiency in some specific factor, and whether it is due to the same cause as the variation in the growth rate, cannot at present be decided. The experimental diet (McCollum 3143) was notably deficient in two nutritional requirements, inorganic phosphate and vitamin D. Coward *et al.* [1932] state that "the reserves of vitamin D in the young rat vary in different individuals and in different litters," basing their conclusion on the variation in the growth of these animals on a vitamin D-deficient, but otherwise adequate, basal diet. The results of the experiments described in this paper are not inconsistent with the view that the observed variations in the calcifying power of the cartilage may, like those in the growth rate, be a result of differences in the vitamin reserves of the animals. Although it is held on good grounds that changes in the composition of the blood are primarily responsible for the deficient calcification in rickets, it is difficult to avoid the conclusion that changes in the calcifying power of the cartilage may also play a part in the story.

SUMMARY.

The calcifying power of the cartilage in the bones of rachitic rats varies with the rate of growth of the animals over a 3 to 4-week period on a rachitogenic diet and decreases as the period on diet is increased.

I should like to thank Prof. R. Robison for much help and valuable criticism.

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DESCRIPTION OF FIGURES IN PLATE II.

Tibia slices stained by v. Kossa's silver nitrate method.

X = calcification *in vitro* in the hypertrophic cartilage zone.
h.c. = hypertrophic cartilage.
s.c.c. = small-celled cartilage.

Figs. 1–3. Table II, Exp. 1, Rat 2. 10 days on a rachitogenic diet.

Figs. 4–6. Table II, Exp. 1, Rat 3. 28 days on a rachitogenic diet.

Fig. 1. Control slice. Note the narrow zone of uncalcified hypertrophic cartilage.

Fig. 2. Slice after immersion for 20 hours in the inorganic solution 8 : 4 : 0 at 37° and p_H 7·4.

Fig. 3. Slice after immersion for 20 hours in the ester solution 8 : 3 : 10. Note the dense band of new calcification extending to the diaphysis in this slice and in that shown in Fig. 2.

Fig. 4. Control slice. Note the breadth of the uncalcified zone.

Fig. 5. Tibia slice after 20 hours in solution 8 : 4 : 0. The very small amount of new calcification (<1) obtained *in vitro* indicates the decreased calcifying power of the hypertrophic cartilage. Note the position of the deposit on the "line."

Fig. 6. Tibia slice from the same rat as (5) after 20 hours in solution 8 : 3 : 10. Calcification is denser and more extensive than in absence of phosphoric ester.

XCVIII. THE CALCIFICATION *IN VITRO* OF KIDNEY, LUNG AND AORTA.

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(*Received March 6th, 1934.*)

ALTHOUGH calcification in the animal body is normally confined to hypertrophic cartilage and osteoid, in certain abnormal or pathological conditions deposits of calcium salts may also be formed in other tissues, among which are the kidney, the lung and the arteries. Some of these conditions, for example hypervitaminosis D, are associated with exceptionally high concentrations of blood-calcium or phosphate, suggesting that the plasma has become by some agency supersaturated with calcium salts. In others degenerative changes in the tissues themselves precede, and may possibly be the primary cause of, calcification.

Without attempting to reproduce *in vitro* all the abnormal conditions concerned it seemed of importance to discover whether the calcification of such tissues could be induced in our experimental solutions, and if so, in what respects the process would differ from that taking place in hypertrophic cartilage and osteoid. Experiments of this kind, though carried out with the tissues of normal animals, might yet throw light on abnormal calcification and, by differentiation, on the normal process of calcification in the skeleton.

Two questions of particular interest were: (*a*) whether the phosphatase which occurs in some of these tissues is at all responsible for their calcification, and (*b*) whether these tissues possess in any degree properties akin to those which constitute the second mechanism of cartilage.

EXPERIMENTAL.

The technique was similar to that employed for experiments on the calcification *in vitro* of cartilage and bone. Calcifying solutions of similar composition were used [Robison and Rosenheim, 1934]. They are referred to as solution 8 : 6 : 10, 8 : 6 : 0, *etc.*, according to their content of calcium, inorganic P and organic P respectively in mg. per 100 ml. of basal salt solution. The organic phosphorus was in the form of α-glycerophosphoric ester.

Our experiments were confined to the kidney, lung and aorta. The tissues were obtained from normal rats and rabbits of various ages and in a few cases from young rachitic rats. They were excised as rapidly as possible, with precautions to avoid bacterial contamination, and small pieces were immersed in the warm calcifying solutions at p_H 7·4 and left at 37° for various periods. Subsequently these pieces were fixed in 10 % neutral formalin, washed, dehydrated and embedded in paraffin. Serial sections were cut, stained with silver nitrate and counterstained. A black deposit of metallic silver formed in the

[1] Grocers' Company Research Student. The work described in this paper formed part of a thesis submitted by A. H. Rosenheim and approved for the degree of Ph.D. in the University of London.

sections on exposure to light was accepted as evidence of the presence of solid phosphate or carbonato-phosphate of calcium, that is, of tissue calcification. The deposits obtained were never so heavy as to be readily apparent before sectioning and staining.

Pieces of each tissue, fixed without immersion in the calcifying solutions, were sectioned and stained as controls.

In some experiments small pieces of the same kidney, lung or aorta were left for different lengths of time in samples of the same calcifying solution, each piece in a separate flask. In other experiments the pieces of each tissue were immersed for the same length of time but in a number of solutions differing in their content of calcium, inorganic phosphate and phosphoric ester.

The results obtained show that calcification of kidney, lung and aorta *in vitro* can be realised under suitable experimental conditions, but that such calcification takes place much more slowly than that of hypertrophic cartilage and is much more erratic in occurrence and distribution.

No attempt has been made to group the results of individual experiments together, since these were often irregular, one piece of tissue possibly showing calcification in 4 days while another piece of the same tissue might fail to calcify in a longer period or in a solution of higher Ca × P level.

The positive and negative results obtained with rat's kidney and lung are collected in Table I and those obtained with the aortae of rats and rabbits in Table II.

From Table I it will be seen that calcification occurred with some regularity in pieces of rat's kidney and lung immersed in solutions containing phosphoric ester, but that the deposits were not generally formed in less than 2 days. In absence of phosphoric ester calcification was only once obtained in kidney and not once in lung, although a large number of experiments were performed with highly supersaturated inorganic solutions and the period of immersion was extended to 4 days.

Table I. *Calcification of kidney and lung* in vitro.

		Inorganic solutions		Solutions containing phosphoric ester			
mg. per 100 ml.	Ca	8	8	8	8	8	8
	P inorg.	5	6	4	5	6	4
	P org.	0	0	10	10	10	30
Tissue	Days						
Kidney	1	– – – –	– – – –	– –	– –	– – –	+
		.	– – – –	.	.	– – –	– –
	2	– –	+	+	–	+	+ +
		.	– –	.	.	.	.
	3	–	–	.	.	.	.
	4	–	–	.	.	.	.
Lung	1	– –	– –	.	–	–	–
	2	–	–	.	–	+ +	+
	3	.	.	.	.	.	+
	4	–	–	.	+	+	.

Each sign in Tables I and II refers to a separate piece of tissue, calcification *in vitro* being indicated by + and absence of calcification by –.

In the aortae both of rats and rabbits, however, calcification did occur in the inorganic solutions, although not invariably and never in less than 3 days (Table II). These solutions were very highly supersaturated and would certainly have produced regular and extensive calcification in cartilage within 10–16 hours.

Table II. *Calcification of aorta* in vitro.

		Inorganic solutions			Solutions containing phosphoric ester			
mg. per 100 ml.	Ca	8	8	8	8	8	8	8
	P inorg.	4	5	6	4	5	6	4
	P org.	0	0	0	10	10	10	30
Species	Days							
Young rat	1	.	−	.	−	.	.	−
	2	.	− −	−	−	.	−	−
	3	.	− −	−	−	.	−	−
	4	.	−	− −	− −	− −	−	.
	5	.	−	− −	−	−	+	.
		.	.	.	.	.	−	.
Full-grown rat	1	.	−	−	−	−	−	.
	2	.	−	.	−	−	−	.
	3	.	−	+	−	.	−	.
	4	.	+	− − −	−	+ + +	+	−
		.	−	.	.	− − −	−	.
	5	.	+	+ +	.	+	+ +	.
		.	.	− − −	.	−	− − −	.
	6	.	−	− −	.	+	+	.
		.	.	.	.	.	− −	.
	7	.	.	+	.	−	.	.
	10	.	.	.	.	.	+	.
Young rabbit	3	.	−	.	.	−	.	.
	4	−	−	.	.	.	.	.
	5	− −	+ +	+	.	− −	−	.
		.	−	.	.	.	.	.
	6	.	+	.	.	−	.	.
Full-grown rabbit	2	.	.	− − (− −)	.	.	−	.
	3	+	− −	− −	.	.	−	.
	4	.	.	+	.	.	.	.
	5	+	+ (+ +)	+	.	−	−	−
		.	− − (− −)	−	.	.	.	.
	6	.	.	+	.	.	.	.
	8	.	+	+	.	.	−	.

Signs in brackets refer to lengths of aorta treated with acetone or desiccated (see text).

In rat's aorta calcification was somewhat more frequent and was denser in presence of phosphoric ester but no such effect was observed in rabbit's aorta. This result was expected, since it has been shown that the former tissue contains phosphatase [Macfarlane *et al.*, 1934], while the latter is devoid of the enzyme [Kay, 1928]. From the results quoted the presence of glycerophosphoric ester would indeed appear to have had some inhibitory effect on the calcification of rabbit's aorta, and this too is reasonable, since the unhydrolysed ester must compete with the inorganic phosphate for calcium ions.

It was noted that the deposits formed in the aorta whether in presence or absence of ester became more extensive and denser as the period of immersion was prolonged and also that the aortae of full-grown rats and rabbits became calcified more readily than those of younger animals. On the other hand, there was no evidence that the age or condition of the animal influenced the calcification of kidney and lung *in vitro*. These tissues were taken from young rachitic rats, young normal rats and adult rats, and positive and negative results were distributed fairly evenly among the three groups.

Effect of acetone and of desiccation on the calcifiability of the rabbit's aorta.

It has previously been shown that the second calcifying mechanism of hypertrophic cartilage is seriously damaged by treatment of the cartilage with acetone or alcohol, or by desiccation [Robison *et al.*, 1930]. Experiments were, therefore, performed to discover whether similar treatment would affect the calcifiability of rabbit's aorta which possesses no phosphatase. The aortae of three full-grown rabbits were used. One portion of each aorta was immersed directly in the calcifying solution while another portion was first soaked in acetone for 22 hours and a third dried over sulphuric acid *in vacuo*. In two experiments solution 8 : 5 : 0 was used and the period of immersion was 5 days. In the third experiment the solution was 8 : 6 : 0 and the period only 2 days. Calcification was obtained in one of the 5-day experiments in the two lengths of aorta which had been treated with acetone and desiccated respectively (Plate III, Fig. 4), but not in the control piece. There was no calcification in any part of the aortae used for the other two experiments. It is clear, therefore, that the calcifiability of the aorta is not diminished but rather enhanced by acetone treatment and desiccation, and that the qualities leading to the calcification of this tissue are markedly different in this respect from the second mechanism of hypertrophic cartilage.

Description of the deposits.

(*a*) *Kidney.* Calcification *in vitro* was usually confined to a narrow zone of cortex beginning a few cell layers from the exterior, though in some cases the extreme peripheral zone was the most densely calcified. The deposits were finely granular and extended over the basement membranes of the tubules and often throughout the tubule cells. The nuclei of these cells also appeared to be calcified (Plate III, Fig. 1). Deposits were frequently observed in the glomeruli and on the glomerular capsules. The distribution of the deposits formed in the rat's kidney *in vitro* bears some resemblance to that of the calcification in the same tissue described by Brand and Holtz [1929] and produced by overdosage with irradiated ergosterol.

Although half kidneys or transverse slices were used in these experiments so that at least one cut surface of the medulla was always exposed to the solution, only slight traces of calcification were observed in this region. In this connection it is of interest that the cortex has been shown by Kay [1926] to possess considerably greater phosphatase activity than the medulla.

(*b*) *Lung.* The deposits obtained in lung were not localised in any special part of the organ. There was diffuse granular deposition, denser in some places than in others and extending irregularly throughout the sections examined. It was not confined to the cells lining the alveoli but appeared also in the alveolar spaces. There was fairly heavy calcification in the walls of the bronchioles (Plate III, Fig. 2).

(*c*) *Aorta.* Calcification *in vitro* in different regions of the same aorta was very varied in amount, being usually densest in the ascending aorta and aortic arch (Plate III, Fig. 3). Its distribution thus corresponded with that of the calcified areas observed in the aorta in experimental hypervitaminosis D in the rat and the rabbit as described by Duguid [1930] and Vanderveer [1931] respectively.

Calcification commenced most often on the outer side of the media or in the adjacent layers of the adventitia; sometimes, however, it occurred in the middle or inner layer of the media, while the outer layers remained free from deposit. Occasionally the calcified zone extended in places almost through the entire

thickness of the vessel wall though adjoining parts of the wall might be free from deposit (Plate IV, Fig. 8).

The elastic fibres in the broad medial coat are the most prominent feature seen in the stained transverse sections of the aortic arch and dorsal aorta of rats and rabbits. The intima is not well developed. The bands of deposit stretched along and between the elastic laminae (Plate IV, Figs. 5 and 7) and the fine granules of calcium salt very often appeared to have been formed on the fibres themselves (Plate IV, Fig. 8). Relatively large rounded aggregates definitely located at intervals along the fibres were sometimes seen (Plate IV, Fig. 6). Wenzel [1928] has observed the deposition of calcium salts in granular form on the elastic fibres in experimental hypervitaminosis D; while deposits in the media, between the elastic laminae, have been described by Kreitmair and Hintzelmann [1928].

Various experiments on calcification of the aorta.

Certain further experiments which were essentially of a preliminary nature may be briefly referred to here.

1. Although there was little evidence of marked degeneration in the tissues except after the most prolonged periods of immersion, less obvious physico-chemical changes must have been taking place from the time of excision. That such degenerative changes might be essential for calcification seemed the more possible in view of the relative slowness with which calcification set in and its sporadic appearance.

In one of the experiments undertaken to investigate this point, an excised rat aorta was kept 3 days at 37° in a solution of Ca × P product too low to produce calcification. Pieces of the tissue were then transferred to the more concentrated solutions 8 : 6 : 0 and 8 : 5 : 10 and left for further periods of 1, 2 or 4 days. Calcification did not occur in less than 4 days after transference of the tissues to the latter solutions. Such degeneration of the tissue as may have occurred during the preliminary period did not, therefore, serve to produce more rapid calcification than usual.

In other experiments sections of aorta were cut on the freezing microtome and alternate sections were stained for fat and calcium salts. Although the stainable fat was found to increase in amount with increasing periods of immersion at 37° there seemed to be no connection between the occurrence and location of fatty degeneration and the occurrence of calcification.

2. With the usual technique the pieces of aorta immersed in the experimental solutions remained in a highly contracted condition very different from that of the aorta *in vivo*. In a few experiments an attempt was made to maintain part of the vessel wall in a distended state. A piece of the aorta was slit open and stretched between the ground ends of two lengths of glass tubing, which were clamped together. A few ml. of 8 : 6 : 10 solution were forced through the tissue drop by drop at a pressure of about 160 mm. Hg over a period of 4 days. During this time solution and tissue were maintained at 37°. Calcification, however, did not take place.

3. Calcification of certain tissues is known to occur as a result of injury. A length of aorta was, therefore, purposely injured by scraping with a sterile scalpel or by pinching with forceps before immersion in the calcifying solutions. Such injury did not lead to increased calcification *in vitro*.

4. Since permeability of the aorta wall may be a factor limiting the rate of calcification and since testicular extract has been shown to exert a marked effect in increasing tissue permeability [McClean, 1931], an experiment was

carried out in which 0·2 ml. of this extract (for which we are indebted to Drs McClean and Morgan) was injected into the media of a rabbit's aorta before excision. There was no evidence of calcification in this aorta after 3 days in 8 : 5 : 0 solution.

Discussion.

If the results of these experiments are to be applied to the problems of pathological calcification it is clear that great caution must be used. In the body deposits of calcium salts are frequently found in abnormal or necrotic tissues whereas the calcification which was obtained *in vitro* occurred in tissues taken for the most part from normal animals. The deposits obtained in the arteries *in vitro* would, indeed, appear to bear less resemblance to those occurring in such conditions as Mönckeberg's sclerosis or atheroma (associated with muscular and fatty degeneration respectively) than to those formed in the early stages of experimental hypervitaminosis D in which tissue degeneration is relatively slight and appears to be of secondary nature [Wenzel, 1928; Vanderveer, 1931]. In the lung and kidney also the calcification *in vitro* was not obviously related to any marked degenerative changes; though doubtless some tissue degeneration must have occurred during the prolonged period of immersion.

There are grounds for assuming that in hypervitaminosis D the condition of supersaturation of the circulating fluid with calcium salts is a determining factor in tissue calcification, as it is in our experimental solutions. In necrotic or degenerate tissues factors of a different type are introduced.

In connection with the etiology of arterial disease it is of some interest that in our experimental solutions calcification occurred in isolated pieces of aorta subjected to no mechanical stresses and not distended by the force of the blood stream.

The experiments described in this paper have shown that in a medium supersaturated with calcium salts, calcification of certain tissues other than hypertrophic cartilage and osteoid may occur without participation of phosphatase, although if the tissue contains this enzyme and phosphoric ester is present the likelihood of deposition will be increased. These results do not suggest that the tissue phosphatases are essential factors in pathological calcification[1]. At least one of the tissues whose calcification was studied, the aorta, possesses qualities which can provoke deposition of calcium salts in the tissue from supersaturated solutions before spontaneous precipitation occurs in the solution itself. By this empirical criterion we should recognise that the aorta possesses the second mechanism, but the slow and erratic formation of the deposits indicates a very low degree of activity. It is not proved that the mechanism in the aorta is identical with that in cartilage. There is, indeed, some evidence to the contrary, for example, in the different effect of acetone on these two tissues. In the aorta the qualities which favour calcification are perhaps associated with the surface of the fibres. It is not unlikely that similar forces at fibrillar interfaces may also assist deposition in cartilage, even if the high activity of the second mechanism in this tissue should ultimately prove to be due to an enzyme complex, as we have suggested elsewhere [Robison and Rosenheim, 1934].

The results of these experiments again emphasise the very special position occupied by hypertrophic cartilage and osteoid with respect to the dual calcifying mechanism. They show also that certain other tissues possess qualities

[1] In heterotopic ossification, in which true osteoid tissue is developed in abnormal locations, phosphatase probably plays the same part as in normal bone formation [Huggins, 1931].

which, although unable to effect their calcification under normal conditions, may do so if the calcium-phosphate level in the blood is abnormally raised. Whether such qualities become further developed as a result of tissue degeneration must be left for future investigation.

SUMMARY.

1. Calcification of the kidney, lung and aorta excised from normal animals has been realised *in vitro* by immersion of the tissues in experimental solutions of similar composition to those used for calcification *in vitro* of hypertrophic cartilage.

2. Calcification of these tissues took place much more slowly than that of hypertrophic cartilage, and especially in the aorta, was erratic in occurrence and distribution.

3. Calcification occurred with some regularity in pieces of rat's kidney and lung immersed in solutions containing phosphoric ester, but not usually in less than 2 days. In absence of phosphoric ester calcification was only once obtained in kidney and never in lung.

4. Calcification occurred, though not invariably, in aortae both of rats and rabbits immersed in very highly supersaturated inorganic solutions for periods of 3 to 8 days.

5. The deposits formed in the kidney, lung and aorta *in vitro* had some resemblance in appearance and distribution to those described as occurring in the same tissues *in vivo* in experimental hypervitaminosis D.

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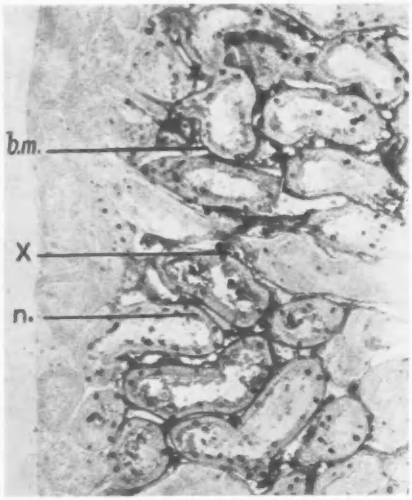

Fig. 1.

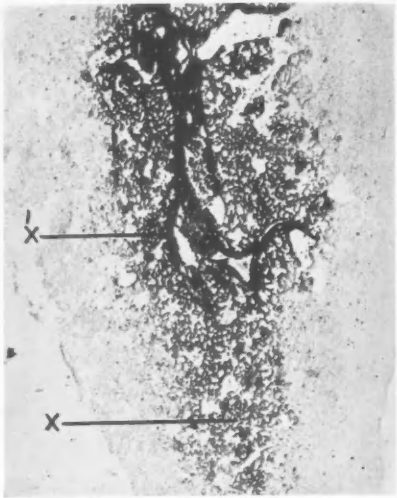

Fig. 2.

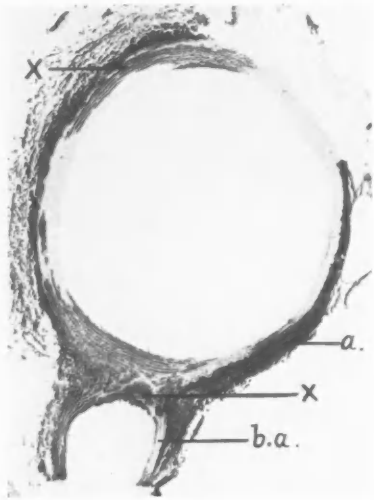

Fig. 3.

Fig. 4.

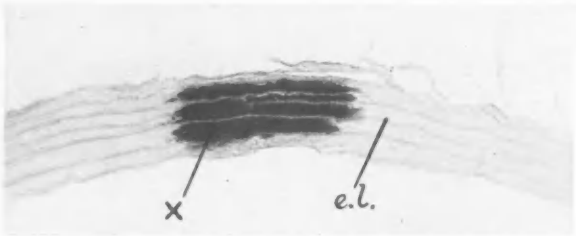

Fig. 5.

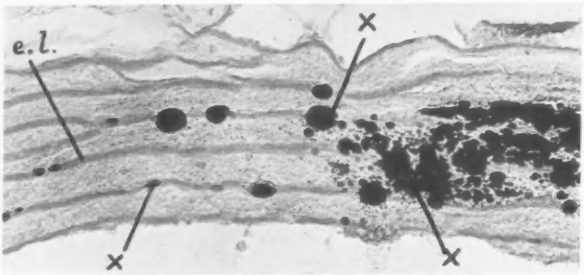

Fig. 6.

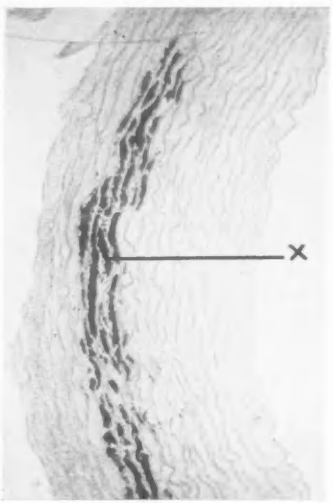

Fig. 7.

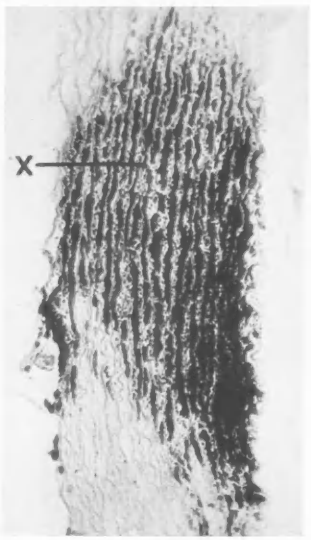

Fig. 8.

DESCRIPTION OF FIGURES IN PLATES III AND IV.

Sections of kidney, lung and aorta, cut after immersion of the tissues in various calcifying solutions for the periods specified. The sections were treated with silver nitrate to show the tissue calcification (*X*) which occurred *in vitro*.

a. = aorta.
b.a. = branch artery.
b.m. = basement membrane of tubule cell.
e.l. = elastic lamina.
n. = nucleus of tubule cell.

PLATE III.

Fig. 1. Cortex of the kidney of a full-grown rat. 2 days in solution 8 : 4 : 30. Note the calcification in the basement membrane of the tubules and in the nuclei of the tubule cells. (Mag. × 250.)

Fig. 2. Lung of the same rat. 4 days in solution 8 : 5 : 10. (Mag. × 27.)

Fig. 3. Transverse section of the aortic arch of a full-grown rat. 4 days in solution 8 : 5 : 10. Note the heavy calcification of the adventitia and media in the main and branch arteries. (Mag. × 22.)

Fig. 4. Aorta of rabbit. The tissue was soaked for 22 hours in acetone and subsequently immersed for 5 days in solution 8 : 5 : 0. The section illustrates the patchy distribution of calcification *in vitro* noted also in tissues not subjected to acetone treatment. (Mag. × 20.)

PLATE IV.

Fig. 5. Thoracic aorta of a full-grown rat. 3 days in solution 8 : 6 : 0. Note the deposit apparently located in the bands of muscle and connective tissue between the elastic laminae. (Mag. × 140.)

Fig. 6. Part of the same aorta as in Fig. 3. 5 days in solution 8 : 6 : 0. Note the rounded aggregates apparently formed in and on the elastic laminae. (Mag. × 350.)

Fig. 7. Aorta of full-grown rabbit. 3 days in solution 8 : 4 : 0. Slight calcification was obtained in the outer half of the media. The deposit is apparently located between the elastic laminae. (Mag. × 140.)

Fig. 8. Aorta of full-grown rabbit. 5 days in solution 8 : 5 : 0. Note the heavy calcification extending almost through the entire thickness of the artery wall. The deposit is chiefly between the laminae, but where it is densest it would seem to overlap them. In places fine granules are visible on the fibres between the bands of deposit. (Mag. × 140.)

XCIX. THE PHOSPHATASE ACTIVITY OF ANIMAL TISSUES.

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The presence of a highly active phosphatase in bone and ossifying cartilage forms the basis of a conception of the process of calcification in these tissues for which much evidence has been accumulated [Robison, 1923]. That calcification does not normally occur in all tissues which contain phosphatase is not necessarily prejudicial to this conception since the animal organism makes wide use of phosphoric esters of varied type and it is not surprising that many of its tissues should contain enzymes by which the synthesis and hydrolysis of these esters can be accomplished. We were, however, interested in the possibility that the presence of phosphatase may be correlated with the calcification which occurs in some of these tissues in certain abnormal bodily states.

The distribution and behaviour of the tissue phosphatases have been the subject of many investigations, which have recently been reviewed by Kay [1932], who discusses the limitations of the experimental methods employed. It has long been recognised that these phosphatases are not all identical, while Akamatsu and his co-workers have shown that more than one type may be present in the same tissue [Akasawa, 1929; Kurata, 1931]. The present experiments were concerned solely with the distribution of the phosphomonoesterase of high optimum p_H which occurs in bone. They were intended to supplement the results already obtained on the relative activities of the tissues of different animal species. It was desired also to eliminate the possibility that a negative or low phosphatase activity reported for any tissue was due simply to lack of magnesium in the tissue or tissue extract. The pronounced effect of magnesium on phosphatase derived from different sources has been described by Erdtman [1927; 1928] and by Jenner and Kay [1931]; the latter concluded that magnesium acts as an accelerator rather than as a coenzyme.

In these experiments we have made a more extended trial of a method which had been found valuable in a study of phosphatase development in rudimentary bones and minute explants of embryonic tissue [Fell and Robison, 1929; 1930]. A number of pieces of each tissue, roughly 1·0–4·0 mg. dry weight, were cut as nearly uniform in size and shape as possible, placed on cover slips and rapidly dried in an evacuated desiccator. The tissue fragments were weighed on a microbalance and placed separately in stoppered tubes of 5 ml. capacity to which 2 ml. distilled water and a small drop of chloroform were added. After standing 24 hours at room temperature, 2 ml. of a 0·2*M* solution of sodium β-glycerophosphate (p_H 8·9) were added, together with a further drop of chloroform; if necessary the p_H was adjusted to 8·6. From one of the duplicate tests carried out on each tissue examined, a sample was then removed for estimation of inorganic phosphate. This 0-hour value, which rarely exceeded 0·004 mg. P

per ml., represented the sum of the inorganic phosphate present in the glycerophosphate solution *plus* that dissolved out of the tissue and was deducted from the final value obtained. The tubes were placed in an incubator for 48 hours at 25° or 24 hours at 37°; at the end of this time the p_H was checked, and suitable samples were removed for estimation of the phosphate by the Briggs method. When the tests were carried out in presence of magnesium this was added as $MgSO_4$, in the glycerophosphate solution, so that the final concentration of Mg was 0·003*M*. This concentration was chosen as the most suitable after preliminary tests on a wide range of tissues. It falls within the limits of optimum concentration found by Jenner and Kay [1931]. No added buffers were used in these experiments since their presence is not without effect on phosphatase activity. Sodium glycerophosphate itself has considerable buffering powers at p_H 8·6; and in more than 90 % of the tests the p_H did not fall below 8·5. Any tests in which the final p_H was lower than 8·4 were rejected. The fall in p_H occurred chiefly in the tests with liver, lung, spleen and muscle and was presumably due largely to glycogenolysis. Since the p_H-activity curve of phosphatase is very steep it may be well to state that differences in the final p_H did not account for the observed variation in the activity of duplicate samples.

The majority of the tests were carried out at 25° for 48 hours, the remainder at 37° for 24 hours. The former conditions usually gave somewhat higher values and were considered preferable owing to the greater stability of the enzyme at the lower temperature.

As a result of many hundreds of tests certain comments may be made on this method.

1. It is specially applicable to investigations in which only minute amounts of tissue are available, as in young or small animals, or in which it is desired to study the localisation of the enzyme in particular zones of the tissue.

2. It gives a direct relationship between the activity and the weight of solid matter in the tissue; the dry weight of tissues with variable water content can be determined with greater accuracy than the wet weight and may provide a more suitable basis for comparison.

3. A large number of tissues from the same animal can be conveniently examined.

4. Although in certain tissues reasonably good agreement was obtained between duplicate samples, in others a wide variation was found, indicating uneven distribution of the enzyme. A limited number of tests may therefore fail to give a fair indication of the average phosphatase activity of a tissue.

5. Since duplicate determinations can only be carried out on separate pieces of tissue it is impossible to check the accuracy of individual values.

6. The assumption that no loss of phosphatase activity occurs during drying may not be equally correct for all tissues, and is difficult to verify exactly in the case of tissues with variable phosphatase content. There was, however, no indication of any serious loss of activity.

7. Because of the variation in individual values, the method is not the most suitable for studying the exact effect of p_H and other factors on the phosphatase activity of different tissues.

As these comments indicate, the method possesses definite advantages for certain purposes but does not replace the usual method in which the moist tissues are ground with chloroform water.

The average values of A/W obtained for tissues of animals of different species are shown in Table I, those for tissues of rats of different ages in Table II. The tissues include some for which no data were previously available. In general

Table I. *Phosphatase activity (A/W) of mammalian tissues. (Average values.)*

A = inorganic phosphate (mg. P) liberated from 0·1 *M* Na β-glycerophosphate in 48 hours at p_H 8·6 and 25°.
W = weight of tissues (mg.).

Final conc. of $MgSO_4$ added ...	None						0·003 *M*					
Animal	Rat	Rabbit	Guinea-pig	Mouse	Cat	Dog	Rat	Rabbit	Guinea-pig	Mouse	Cat	Dog
No. of animals examined ...	3	3	1	2	1	1	3	3	1	2	1	1
Age of animals (weeks) ...	4	6–8	4	12	9	5	4	6–8	4	12	9	5
Tissue												
Femur epiphysis	0·25	0·22	—	—	—	—	0·60	0·56	—	—	—	—
Costochondral junction	0·28	0·53	—	—	0·88	0·73	1·10	1·10	—	—	3·18	2·40
Rib cartilage	<0·01	<0·01	<0·01	<0·02	0	0	0·02	<0·01	—	<0·02	0·01	0·03
Trachea	0·03	<0·01	<0·01	<0·02	0·03	0·02	0·12	0·02	0·06	0·02	0·14	0·27
Intestinal mucosa	—	0·43	2·1	0·67	3·0	2·6	—	1·13	2·42	6·77	7·75	11·8
Kidney cortex	0·38	0·16	0·32	0·66	0·46	0·41	0·55	0·22	0·79	2·70	1·50	1·4
Bladder	0·07	0·06	0·07	0·13	0·06	0·03	0·16	0·16	0·13	0·50	0·35	0·08
Aorta	0·09	<0·01	<0·01	<0·01	0	0	0·38	<0·01	<0·01	0?	0?	0
Dorsal aorta	0·09	<0·01	—	0	—	0	0·38	<0·01	—	0	—	0
Cardiac muscle	—	0·01	0·03	0·05	0·01	<0·01	—	0·02	0·05	0·09	0·04	0·02
Skeletal muscle	<0·01	—	0·02	0·03*	0·01	—	—	—	—	0·10*	0·01	0·01
Lung	0·17	0·07	0·10	0·06	0·11	0·05	0·61	0·20	0·25	0·10	0·21	0·28
Liver	0·03	0·06	0·02	0·03	0·02	0·06	0·05	0·12	0·04	0·06	0·07	0·26
Spleen	—	0·07	—	0·03	0·03	0·09	—	0·21	—	0·15	—	0·36
Pancreas	—	0·03	—	0·03	0·02	0·11	—	0·06	—	0·04	0·03	0·36
Thyroid	0·16	0·02	0·03	0·12	0·02	0·01	0·41	0·05	0·13	0·30	0·07	0·04
Adrenal	0·09	0·03	0·33	0·02	0·06	0·03	0·26	0·05	0·73	0·05	0·29	0·11
Thymus	—	0·06	—	0·03	—	0·11	—	0·07	—	0·10	—	0·48

* Intercostal muscle.

Table II. *Phosphatase activity (A/W) of tissues of rats of different ages. (Average values.)*

A = inorganic phosphate (mg. P) liberated from 0·1 *M* Na β-glycerophosphate in 24 hours at p_H 8·6 and 37°.
W = weight of dried tissue (mg.).

Final conc. of $MgSO_4$ added...	None			0·003 *M*		
Age of rat	1 day	4 weeks	6 weeks	1 day	4 weeks	6 weeks
No. of rats examined ...	2	3	2	2	3	2
Tissue						
Femur epiphysis	0·23	0·21	0·18	1·23	0·48	0·57
,, junction	—	0·48	0·33	—	1·04	0·79
,, shaft	0·36	0·07	0·07	1·45	0·27	0·16
Rib bone	—	—	0·14	—	0·31	0·30
Costochondral junction	0·20	0·17	0·18	0·77	0·40	0·52
Tooth point	0·36	0·09	0·06	1·04	0·25	0·13
,, root		0·41	0·25		1·18	0·59
Membrane bone	0·40	0·08	0·10	1·26	0·57	0·23
Rib cartilage	<0·01	<0·01	<0·01	0·02	0·01	0·01
Trachea	0·03	0·03	0·04	0·25	0·16	0·24
Intestinal mucosa	0·67	—	0·80	2·94	2·06	2·42
Kidney cortex	0·16	—	0·34	0·37	0·38	0·68
,, medulla		—	0·04		0·11	0·15
Bladder	0·06	0·08	0·05	0·20	0·19	0·27
Aorta	0·08	0·02	0·14	0·11	0·45	0·86
Cardiac muscle	—	—	0·06	—	0·10	0·10
Skeletal muscle	<0·01	<0·01	<0·01	0·08	0·01	0·02
Lung	0·29	0·12	0·10	0·42	0·24	1·08
Liver	0·02	—	0·02	0·05	0·04	0·03
Spleen	0·05	—	0·02	0·20	0·07	0·09
Brain	0·06	—	0·04	0·10	0·06	0·07
Thyroid	0·05	0·04	0·04	0·38	0·21	0·42
Adrenal	0·04	0·03	0·06	0·28	0·20	0·23
Thymus	0·02	—	0·02	0·07	0·06	0·08

the relative activities are in agreement with those obtained by other methods. In nearly all cases the activity was decidedly increased in presence of magnesium. High values are shown for intestinal mucosa, kidney cortex, bones and teeth; with regard to the last two, it should be remembered that a large proportion (50–66 %) of the dry tissue fragment consisted of inorganic material, so that the A/W of the organic portion was actually much higher than the value shown. Rib cartilage and skeletal muscle were almost inactive in all species examined, even in the presence of magnesium, but cardiac muscle had a slight but definite activity. Most of the remaining tissues gave intermediate values, rather variable from one species to another. The definite activity of the bladder in all species may be noted [*cf.* Huggins, 1931] and also that of the trachea in the rat, cat and dog. Calcification of the tracheal cartilage occurs not infrequently *in vivo*, especially in old age; and the presence of phosphatase may seem significant when it is recalled that in the rudimentary bone the appearance and increasing activity of this enzyme runs parallel with the hypertrophy of the cartilage cells.

The examination of the aorta was carried out with special interest, in view of the frequency with which this tissue becomes calcified in certain abnormal states. Among the species examined, the aorta of the rat alone showed definite phosphatase activity; and in this respect the contrast between the rabbit and the rat is remarkable. Calcification of the aorta has been frequently observed *in vivo* in both rat and rabbit, and calcification has also been induced *in vitro*

in the excised aortae of both animals [Rosenheim and Robison, 1934]. While therefore the phosphatase present in the rat aorta may affect in some degree the incidence or extent of such calcification, it would seem that the enzyme cannot be the crucial factor in this process.

Summary.

A method of estimation of the phosphatase activity of tissue fragments is described in which the activity is related to the dry weight of the tissue. The application of this method is discussed.

The values obtained by this method for the phosphatase activity of various tissues in presence and absence of magnesium are given for the rat, rabbit, guinea-pig, mouse, cat and dog. The results generally confirm those previously obtained.

The bladder shows definite phosphatase activity in all species examined; the trachea also showed some activity in most species.

The aorta of the rat was exceptional in possessing a definite phosphatase activity, while in the other species this tissue was practically devoid of the enzyme.

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C. THE EFFECTS OF THE INJECTION OF CERTAIN SALTS ON THE CALCIUM, MAGNESIUM AND INORGANIC PHOSPHORUS OF THE SERUM OF THE RABBIT.

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Brookfield [1933] showed that in the serum of the rabbit there was an inverse relationship between calcium and magnesium, a direct relationship between magnesium and inorganic phosphorus and an inverse relationship between calcium and inorganic phosphorus, the latter of which had been previously observed by Dupré and Semeonoff [1931] and Bourne and Campbell [1932]. The rises and falls in the concentrations of the three ions in the serum were correlated with their relative amounts in the diet. The relationships seemed worthy of further study under other experimental conditions, in order to determine whether the effects observed with diets varying in their mineral contents were of more general significance, and accordingly another series of experiments has been carried out.

Solutions of salts containing Ca^{++}, Mg^{++} and $PO^{=}_{4}$ ions have been injected subcutaneously into rabbits and their effects on the calcium, magnesium and inorganic phosphorus of the serum observed.

Experimental.

Experiments were made on fully grown male rabbits maintained on a diet of cabbage and oats, which (with one exception) had fasted for about 18 hours. Calcium gluconate, laevulate and lactate, magnesium sulphate and lactate and disodium hydrogen phosphate were the salts employed, while control experiments were carried out with sodium chloride and sodium sulphate. The salts, dissolved in the minimum volume of water, were injected subcutaneously into the flank. 5 ml. of blood were withdrawn from the lateral vessel of the ear immediately before the injection and on three separate occasions afterwards. The effects were observed over periods varying from $1\frac{1}{2}$ to 6 hours. The methods of estimating calcium, magnesium and inorganic phosphorus were the same as those used in the dietary experiments [Brookfield, 1933].

The influence of two factors called for control before the results of the experiments could be interpreted; firstly, the effects of four small haemorrhages recurring at relatively short intervals and secondly, the effects of the injection of a solution of an indifferent salt.

1. *The effects of bleeding.* (*a*) *Successive small haemorrhages.* 5 ml. of blood were removed from a rabbit four times at half-hourly intervals; 9 days later the same procedure was repeated, but at hourly intervals and after a further 22 days the animal was bled again, this time at 2-hourly intervals. In the

first experiment the haemoglobin percentage was unaltered at the fourth bleeding; in the other two experiments a fall of 8 % was noted. The serum-calcium, magnesium and inorganic phosphorus values are recorded in Table I.

Table I. *Serum-calcium, magnesium and inorganic phosphorus. Small haemorrhages at short intervals.*

Rabbit 20. Weight 2010 g. 4 bleedings of 5 ml. on each occasion.

Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
½-hourly intervals		
16·55	1·952	3·152
15·70	1·694	2·626
15·15	1·597	2·551
14·95	1·590	2·716
1-hourly intervals		
13·29	2·886	4·382
13·29	2·886	4·382
12·94	2·794	4·762
12·72	2·749	4·794
2-hourly intervals		
13·53	3·615	3·750
13·53	3·542	3·936
13·90	3·357	4·521
13·67	3·204	4·654

It will be seen that the magnesium value tends to fall, but at a very slow rate. The calcium figures show variable results, depending on the time interval. The four bleedings at half-hourly intervals have caused a fall of 1·6 mg. (10 %). Hourly bleedings cause a slighter fall (4 %) and 2-hourly bleedings none. A fall in the inorganic phosphorus followed by a rise after one hour results from bleeding at intervals of half an hour, while with hourly or 2-hourly bleedings the inorganic phosphorus rises.

(*b*) *Progressive changes during a single haemorrhage.* The rapidity with which the fall in the serum-calcium takes place is illustrated by the results recorded in Table II.

Table II. *Serum-calcium and magnesium values during bleeding. Blood taken in two samples of 5 ml. each.*

Rabbit no.	Time of bleeding	Calcium mg./100 ml.	Magnesium mg./100 ml.
19	11.45–12.15	15·28	1·722
	12.15–12.45	14·44	1·694
21	4.30–4.45	15·73	1·895
	4.45–5.0	15·35	1·886
19	10.45–11.0	12·60	1·907
	11.0–11.10	12·25	1·896
20	11.15–11.25	14·35	1·963
	11.25–11.30	14·25	1·951

Each animal was submitted to a single haemorrhage and the blood collected in two successive 5 ml. samples. In one experiment where the time of bleeding was rather prolonged, a fall of 0·8 mg. (6 %) was noted. The effects of haemorrhages on the serum-calcium of rabbits have been referred to by several writers. A decrease of 10 % or more has been reported when observations have been made

at a short interval after the haemorrhage by Stransky [1915], Clark [1920], Stewart and Percival [1927] and Culhane [1927]. Seven hours after bleeding, Moritz [1925] found a slight and inconstant decrease in the serum-calcium value. A fall in the magnesium also was noted by Stransky. These results show that the variations in the concentrations of calcium, magnesium and inorganic phosphorus caused by bleeding are so slight as to have little or no significance in the interpretation of the findings in the injection experiments, which will now be discussed.

2. *The effects of the injection of an indifferent salt.* Sodium chloride was injected in an amount somewhat in excess of those of the other salts employed. The results are recorded in Table III.

Table III. *Injection of sodium chloride. Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 24. Weight 2300 g. 5 ml. bleedings. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	13·29	2·786	3·820
0·36 g. NaCl (0·0062 g. equivalent per kg. in 9 ml. water injected subcutaneously).			
¼	13·29	2·806	4·154
1	12·67	2·726	3·434
4	13·06	2·557	3·701

There is a slight fall in all three constituents after one hour with a tendency on the part of the calcium and inorganic phosphorus to return to their initial values after 4 hours. These changes are unimportant in comparison with the results which follow, and they show that probably any effect observed in the injection of salts containing Ca^{++}, Mg^{++}, or $PO^{=}_{4}$ was to be attributed to these ions themselves.

3. *The injection of solutions containing the Ca^{++}, Mg^{++}, $PO^{=}_{4}$ and $SO^{=}_{4}$ ions.* An attempt was made, in each case, to introduce an amount of salt sufficient to cause a marked effect on the several blood-constituents under review without producing untoward symptoms. The success achieved was only partial. No symptoms were noted after the injection of the calcium salts. Slight ataxia of the hind-limbs resulted from the injection of magnesium sulphate and lactate. Definitely toxic effects were observed however with disodium phosphate and sodium sulphate. In both cases there was some degree of collapse after the injection. The animal injected with disodium phosphate showed marked muscular twitching but ultimately recovered. The animal which received sodium sulphate was found dead 2 days later. No urine was passed during the period of any of the experiments except by the rabbit which received sodium sulphate. None of the animals suffered from diarrhoea.

Results.

For purposes of comparison the amounts of the salts have been expressed as g. equivalents per kg. body weight. The results of the injections of the calcium salts are set out in Table IV, of the magnesium salts in Table V, of sodium sulphate in Table VI and of disodium phosphate in Table VII.

1. *The interrelations of calcium and magnesium.* In each of the 4 experiments where calcium salts have been injected approximately equivalent amounts of calcium have been given. A moderate increase in the serum-calcium has been the result amounting on the average to 3·0 mg. or 23 % of the initial value.

The rise to the maximum takes place within one hour and is followed by an almost equally rapid fall. The rise in the serum-calcium is accompanied by a progressive fall in the serum-magnesium. In the case of the injection of calcium gluconate, there may be a slow return towards normal at 6 hours. The average fall amounts to 1·0 mg. or 37 % of the initial value though it should be noted that in 3 of the experiments (Table IV) the fall appears still to be continuing at the time of the final observation.

Table IV. *Injection of calcium salts. Serum-calcium, magnesium and inorganic phosphorus values.*

	Salt injected	Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
Rabbit 19. 2520 g. Fasting	Gluconate (0·6 g.≡0·0028 g. equiv. Ca/kg.)	0	13·17	2·389	3·720
		½	16·13	1·896	3·861
		2	14·91	1·590	4·106
		6	14·32	1·748	4·152
Rabbit 23. 2000 g. Fasting	Gluconate (0·75 g.≡0·0035 g. equiv. Ca/kg.)	0	13·29	3·091	4·826
		½	16·59	2·567	4·700
		1	16·75	2·097	4·609
		1½	17·77	1·896	4·700
Rabbit 23. 2200 g. Fasting	Lævulate (0·6 g.≡0·0039 g. equiv. Ca/kg.)	0	12·27	3·044	4·252
		½	15·19	2·635	3·990
		1¼	14·57	2·136	—
		2¼	13·48	1·860	—
Rabbit 23. 2170 g. Fasting	Lactate (0·46 g.≡0·003 g. equiv. Ca/kg.)	0	14·46	2·332	2·977
		½	17·02	1·902	3·106
		1	14·97	1·620	2·927
		2	14·82	1·544	3·247

Though the magnesium salts have been injected in amounts equivalent to those of calcium the effects have been considerably greater. The rise in the serum-magnesium averages 5·6 mg. over the initial value, as compared with a rise of 3·0 mg. in the calcium value caused by injections of corresponding amounts of calcium salts. This rise takes place rapidly but is more sustained than in the case of calcium, and it is clear that either the rate of absorption of magnesium salts is much less rapid than that of calcium salts, or alternatively and more probably

Table V. *Injection of magnesium salts. Serum-calcium, magnesium and inorganic phosphorus values.*

	Salt injected	Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
Rabbit 19. 2680 g. Fed with cabbage and oats	Sulphate (0·37 g.≡0·003 g. equiv. Mg/kg.)	0	14·69	2·109	2·779
		¾	11·32	8·078	2·080
		1¼	10·74	8·258	1·890
		4¾	13·31	3·634	3·086
Rabbit 25. 2750 g. Fasting	Lactate (0·37 g.≡0·0031 g. equiv. Mg/kg.)	0	12·62	2·348	3·682
		¾	11·85	6·726	3·262
		1¾	10·54	7·466	3·147
		5	10·61	5·833	3·608

that the removal of excess magnesium from blood is much less readily accomplished. Where magnesium sulphate has been injected (Table V) the serum-magnesium is approaching the normal value after 5 hours, but at the same interval after the injection of the equivalent amount of magnesium lactate (Table V) the serum-magnesium is nearly 3 times greater than the initial value.

These increases in the magnesium concentration have resulted in a marked fall in the calcium value in every experiment. This fall in calcium has been more rapid and greater in extent (4·0 mg. or 27 % of the initial value), following the injection of magnesium sulphate (Table V). The calcium value in this experiment is approaching the initial level after 6 hours. Following the injection of magnesium lactate the serum-magnesium continues at a high level for a considerable time and there is a correspondingly prolonged depression of the serum-calcium, which after 5 hours is still at the low level of 10·6 mg.

The inverse relationship found to exist between calcium and magnesium in the dietary experiments is therefore borne out by the results of these subcutaneous injections of calcium and magnesium salts.

Previous observations of the effects of the injection of calcium and magnesium salts on the calcium and magnesium content of serum are fragmentary and in some cases conflicting. Salvesen *et al.* [1924, 2] found that the magnesium content of the blood of dogs was depressed by the intravenous injection of calcium chloride. Stransky [1915] and Meneghetti [1927] observed a fall in the serum-calcium of rabbits after the injection of magnesium sulphate, the latter worker also showing that the diffusible calcium was increased. A number of observers have shown that the injection of magnesium salts leads to an increase in the calcium excretion and *vice versa*. Thus Mendel and Benedict [1909] found that the injection of calcium chloride into dogs and rabbits led to loss of magnesium, while the injection of magnesium sulphate and chloride increased the excretion of calcium. Greenwald and Gross [1925] noted a temporary increase in the excretion of magnesium in dogs injected with calcium chloride. After introducing metallic magnesium under the skin of rabbits, Reding and Slosse [1923] noted a considerable increase in the output of calcium, and loss of calcium was also observed by Schiff [1920] and by Schiff and Stransky [1920] in infants injected with magnesium sulphate. While a fall in the serum-calcium, accompanied by an increased excretion of calcium, was noted by Pribl [1929] in rabbits after the injection of magnesium sulphate, a rise in the serum-calcium value with calcium retention following the injection of magnesium lactate was reported. Condorelli's [1926] findings are also at variance with those recorded above, in that he observed a considerable elevation of the serum-magnesium following the injection of calcium lactate into rabbits.

2. *The effects of the sulphate ion.* One possible complicating effect in the experiment with magnesium sulphate is the presence of the sulphate ion, for the fall in the calcium value was less when magnesium was introduced as the lactate. Since the injection of phosphate depresses the serum-calcium it was possible that the injection of sulphate might have had a similar effect, and this was controlled in an experiment in which sodium sulphate was injected in amount equivalent to twice that of the magnesium sulphate used. Table VI shows that this has

Table VI. *Injection of sodium sulphate (1·0 g. Na_2SO_4, $10H_2O \equiv 0{\cdot}006$ g. equiv. Na/kg.). Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 23. Weight 2500 g. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	14·34	2·629	4·700
½	13·52	2·629	3·536
1¾	12·20	2·505	3·106
4	11·20	2·557	3·572

resulted in a considerable fall in the serum-calcium but one different in character from that caused by the injection of magnesium sulphate. The fall is much more gradual, and although still continuing at the time of the last observation, 4 hours after the injection, is less than that produced by half the equivalent amount of magnesium sulphate (Table V). It is of interest that Stransky [1915] found a much increased calcium excretion after the injection of sodium sulphate into a rabbit. Table VI shows that while sodium sulphate has no effect on the serum-magnesium, the serum-inorganic phosphorus is depressed by 1·6 mg. (34 %) to rise again after one and three quarter hours, when it moves in a direction opposite to that of calcium.

3. *The interrelations of calcium and inorganic phosphorus.* The changes in the serum-inorganic phosphorus after the injection of the three calcium salts (Table IV) are so slight that it appears that injected calcium has no direct effect on the inorganic phosphorus. When, however, disodium phosphate is injected there is an immediate fall in the serum-calcium (Table VII). In this experiment the phos-

Table VII. *Injection of disodium hydrogen phosphate (0·57 g. Na_2HPO_4, $12H_2O \equiv$ 0·0047 g. equiv. P/kg.). Serum-calcium, magnesium and inorganic phosphorus values.*

Rabbit 19. Weight 2300 g. 5 ml. bleedings. Fasting.

Time after injection (hours)	Calcium mg./100 ml.	Magnesium mg./100 ml.	Inorganic phosphorus mg./100 ml.
0	12·97	2·529	4·121
1	11·39	2·308	10·01
2	10·74	2·423	10·51

phate was injected in amount equivalent to 1·5 times the amounts of the salts used for the calcium injections. The rise in the serum-inorganic phosphorus was rapid and relatively prolonged: at the end of one hour it was 5·9 mg. (140 %) above the initial value, and at 2 hours was at a slightly higher level. At the same interval after the injection, the serum-calcium had fallen by 2·2 mg. or 17 %. Samples were unobtainable after 2 hours owing to the collapsed state of the animal.

From these results it is plain that the inverse relationship between the calcium and inorganic phosphorus of serum observed in the dietary experiments is only partially maintained when calcium or phosphate is injected. The results of the injection of phosphate are in accord with the dietary experiments inasmuch as the serum-calcium falls as the inorganic phosphorus rises. Several previous workers have reported a depression of the serum-calcium on injecting phosphate. Binger [1917] produced a fall of the serum-calcium to 6 mg./100 ml. with the development of tetany by injecting dogs intravenously with disodium phosphate. Tisdall [1922] repeated his experiments with a similar effect, while Salvesen *et al.* [1924, 1] obtained the same results when phosphates were administered orally. The calcium thus displaced from the serum is apparently largely excreted since Greenwald and Gross [1925] found a rise in the calcium excretion in dogs after the injection of neutral sodium phosphate, while Boyd *et al.* [1930] observed, in addition to a fall in the serum-calcium, an increased urinary excretion of calcium after injecting sodium glycerophosphate into the same animals.

In the present investigation injection of calcium has produced no definite fall in the serum-inorganic phosphorus, so that in this respect there is no effect analogous to the inverse relationship of the dietary experiments. The results of other workers are somewhat variable. Bomskov [1930] found that a fall in the

inorganic phosphorus followed the injection of calcium gluconate into rabbits. Salvesen *et al.* [1924, 2] and Collip [1926], using dogs, reported a small rise in the inorganic phosphorus value on injecting calcium chloride. Condorelli [1926] found a greater rise in the rabbit injected with calcium lactate. Greville [1931] found the inorganic phosphorus of the serum of two rabbits slightly elevated after the intravenous injection of calcium laevulate, while Apitzsch [1931] observed that the serum-inorganic phosphorus of human subjects was raised after the oral administration of calcium lactate. Thus the balance of evidence suggests that there is no disappearance of phosphorus from the blood, though it is noteworthy that Greenwald and Gross [1925] found an increased excretion of phosphorus in the dog after the injection of calcium chloride.

4. *The interrelations of magnesium and inorganic phosphorus.* Following the injection of magnesium (Table V) there is a fall in the serum-inorganic phosphorus amounting, after the injection of the sulphate to 0·9 mg. (32 %) and after that of the lactate to 0·5 mg. (15 %). The somewhat greater effect of magnesium sulphate is possibly accounted for by the synergic action of the sulphate ion which itself lowers the serum-inorganic phosphorus (Table VI). It will be seen that the injection of phosphate (Table VII) caused no appreciable change in the serum-magnesium, just as the injection of calcium caused no effect on the serum-inorganic phosphorus. These results are not in accord with the direct relationship between magnesium and inorganic phosphorus observed in the dietary experiments.

Discussion.

The injection experiments differ from the earlier dietary experiments in two important respects which may account to some extent for some of the divergences in the results of the two series. Firstly, whereas in the injection experiments salts were introduced subcutaneously and rapidly absorbed into the circulation, in the dietary experiments much smaller amounts of the relevant ion were slowly absorbed from the intestine over a period occupying 2 hours or more. Secondly, while two ions only were concerned in the injection experiments, a number of ions were being absorbed simultaneously in the dietary experiments, as well as other constituents which may themselves exercise important effects.

These differences do not appear to be of sufficient importance materially to affect the relationship existing between the calcium and magnesium of serum, which in both series of experiments is inverse. It is noteworthy that the effects of the injections have not been as drastic as might be anticipated. In the dietary experiments the rises and falls in the serum-calcium corresponded with similar rises and falls in the serum-magnesium, whereas in the injection experiments the greater and more rapid increase in the concentration of the one ion in the blood has produced a fall in the other hardly greater than those seen in strictly physiological conditions. It is evident that there exist mechanisms whereby the organism can resist attempts to lower a blood constituent to an undesirable level.

When the relations of calcium and magnesium to inorganic phosphorus are studied, differences in the results of the two series of experiments are at once apparent. Although the injection of phosphate causes a fall in the serum-calcium the converse is not observed, since when a calcium salt is injected, the serum-inorganic phosphorus is virtually unaffected. The inverse relationship between the serum-calcium and inorganic phosphorus as observed in the dietary experiments therefore appears to operate in one direction only. The direct relationship between magnesium and inorganic phosphorus observed in the dietary experiments receives no support from the results of the injections, since while the

injection of phosphate leaves the serum-magnesium unaffected, the injection of magnesium salts depresses the inorganic phosphorus so that the two constituents are actually in inverse relationship.

The lack of reciprocity between calcium and inorganic phosphorus on the one hand and between inorganic phosphorus and magnesium on the other which is indicated by the results of the injection experiments appears to find an explanation when the relative concentrations of these constituents in the blood are considered. Normally the calcium of serum is more than equivalent to the inorganic phosphorus, so that it might be anticipated that, when more phosphate was introduced into the blood-stream, combination with calcium would occur with the deposition in some suitable site of calcium phosphate and a consequent fall in the serum-calcium value. Since calcium is present in excess of inorganic phosphorus, it is hardly surprising that the introduction of further calcium produces no fall in the inorganic phosphorus. Similarly, the inorganic phosphorus of serum is more than equivalent to the magnesium, so that, when more magnesium is introduced, it seems reasonable to expect that some of the magnesium will combine with inorganic phosphorus, the disappearance of which from the blood will lead to a fall in the serum-inorganic phosphorus. On the other hand, the addition of further phosphate when inorganic phosphorus is already in excess need hardly be expected to cause a fall in the serum-magnesium.

The injection experiments suggest that fluctuation of the serum-inorganic phosphorus is the chief controlling factor in the calcium-inorganic phosphorus relationship observed in the dietary experiments. This is in harmony with the explanation suggested by Fraser [1932] who regards the relationship as dependent on the periodical liberation of calcium phosphate from the bones. The calcium plays a passive rôle, in that calcium deficiency causes calcium phosphate to leave the depots with consequent rise in the serum-inorganic phosphorus value, whereas the presence of a sufficiency of calcium in the blood leads to a discontinuance of the process, so that the serum-inorganic phosphorus tends to fall. It is obvious that the depots must normally be replenished from time to time and it may fairly be assumed that a rise in the serum-inorganic phosphorus is instrumental in this effect leading to a deposition of calcium phosphate in the bones. Regarded from this aspect, the mechanism of the reciprocal relationship might be stated thus: A rise in the serum-inorganic phosphorus leads to a fall in the serum-calcium by deposition of calcium phosphate in bone, while a fall in the phosphorus value, as by excess of excretion over intake, allows liberation of calcium phosphate from the bones to proceed with consequent rise in the serum-calcium value.

Summary.

1. With a view to confirming earlier observations of the effects of the inorganic constituents of diet on the relationships between the concentrations of calcium, magnesium and inorganic phosphorus in the serum of the rabbit, solutions containing the appropriate ions have been injected subcutaneously and their effects on the three constituents compared with those noted previously.

2. The inverse relationship existing between the concentrations of calcium and magnesium in the serum, first observed when rabbits are kept on certain diets, has been confirmed by the subcutaneous injection of calcium and magnesium salts.

3. The inverse relationship between the serum-calcium and inorganic phosphorus and the direct relationship between the serum-magnesium and inorganic phosphorus is only supported in so far as the injection of disodium phosphate

causes a fall in the serum-calcium. The injection of the gluconate, laevulate and lactate of calcium has no constant effect on the serum-inorganic phosphorus, nor does the injection of phosphate affect the serum-magnesium. The injection of the sulphate and lactate of magnesium depresses the serum-inorganic phosphorus, and the relationship is therefore the converse of that observed in the dietary experiments.

4. The above findings are discussed in relation to the concentration of calcium, magnesium and inorganic phosphorus, normally existing in the blood.

5. The injection of sodium sulphate depresses the serum-calcium and inorganic phosphorus but leaves the serum-magnesium unchanged.

6. The injection of sodium chloride has no significant effect on the serum-calcium, magnesium or inorganic phosphorus.

7. Small haemorrhages cause a slight temporary depression of the serum-calcium.

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CI. DIRECT FERMENTATION OF MALTOSE[1].

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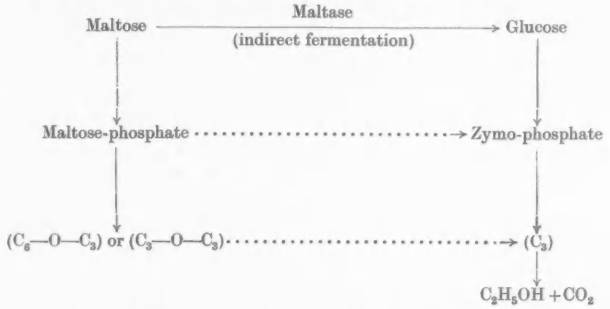
Willstätter designated as "direct fermentation" of disaccharides the fermentation of sugars like sucrose, maltose or lactose without preceding hydrolysis into monosaccharides. The assumption of such direct fermentations conflicts with the prevailing hypothesis that any glycolysis of polysaccharides must be preceded by cleavage of the glucosidic linkages. This classical hypothesis, perhaps first formulated by Emil Fischer and Lintner, has been generally accepted, as it seems to be supported by ample experimental evidence, such as the correlation of fermentability and non-fermentability of carbohydrates by yeasts and other micro-organisms with the presence or absence of specific carbohydrases.

The validity of this theory was questioned by Willstätter and Steibelt [1921]. They found that brewer's yeast generally contained ample maltase to accomplish the conversion of maltose in the wort into glucose for subsequent fermentation. Table I gives a synopsis of the time required for half fermentation and for half hydrolysis of maltose under comparable standard conditions for various yeasts studied in this laboratory and including some examples given by Willstätter and Steibelt [1921] and Willstätter and Bamann [1926, 1]. Munich brewer's yeast (Löwenbräu) ferments maltose rapidly, but can hydrolyse it still faster. With various distiller's yeasts, the time of half hydrolysis is of the same order of magnitude as that of half fermentation, but the maltase content seems to be less than sufficient. A third group, comprising yeast M of the Berlin Institut für Gärungsgewerbe, Vienna distiller's yeast, and New York Lager yeast, is distinguished by a paradoxical lack of maltase. The amount of maltase found is negligible. Although the determination of maltase in such small amounts is not very accurate, even the lowest values for half hydrolysis (highest maltase values) are many times larger than the time required for half fermentation of the same quantity of maltose. The maltase content is far from sufficient to supply the zymase with those amounts of glucose necessary, according to the theory, for the maintenance of the observed swift development of carbon dioxide and alcohol. Willstätter and Oppenheimer [1921–22] observed an analogous discrepancy between the fermentation of lactose and the low amount of lactase in a strain of *Saccharomyces fragilis* Jørgensen.

Before discussing various attempts to reconcile these facts with the classical theory, one might define "direct fermentation" in the light of the present knowledge regarding the zymase complex. The absence of disaccharidase, *e.g.* maltase, as shown by the absence of glucose from the fermenting mixture, leads to the assumption that the sequence of intermediaries in disaccharide fermentation converges at a later stage with that of common hexose fermentation. Thus, we may suspect the existence of disaccharide-phosphates and possibly the transi-

[1] Read before the Section of Biological Chemistry at the Semiannual Meeting of the A. Chem. Soc., Chicago, September 1933.

tory formation of C_6—O—C_3 or C_3—O—C_3 derivatives. The eventual cleavage of the glucosidic linkage in any such molecule could hardly be considered as "maltase" action. The further course of direct fermentation is indicated by the dotted arrows in the diagram.



In order to preserve the classical conception, it may be argued, (1) that the competitive inhibition by glucose, the product of hydrolysis, impairs maltase action in the maltase determination, in contrast to conditions during fermentation when the product of hydrolysis is constantly removed; (2) that the hydrolytic enzyme is regenerated during fermentation; (3) that in these paradoxical cases the method of maltase determination fails to give the full value of maltase activity. The maltase might be present, but not in an extractable form; the active group of the maltase might even be linked with the same colloidal carrier as that component of the zymase system which initiates glucose fermentation, and the glucose molecule would be handed over by a conveyor-like mechanism from the maltase to the zymase without the possibility of glucose being detected or isolated. While such a mechanism would still fall under the definition of direct fermentation, positive proof for it could only be adduced again by the separation of such a "malto-zymase" into maltase and zymase.

Point (1) can easily be refuted since the affinity of glucose for maltase is quantitatively known; thus, the inhibitory effect of the monosaccharide on the enzyme can be evaluated and shown to be of no consequence in comparison with the observed divergencies. Point (2) was raised by Euler and Brunius [1926], when Willstätter and Lowry [1925], and Willstätter and Bamann [1926, 1] succeeded in demonstrating direct fermentation of sucrose and of maltose with yeasts depleted of the respective disaccharidases by treatment with mineral acid or alkali hydroxide. Sucrase determination after fermentation sometimes showed increments, but not sufficient to account for the rate of fermentation. In some instances, *e.g.* in the case of a lactose-fermenting yeast, the enzyme content after fermentation was just as low as before fermentation. No galactosazone, but only lactosazone, could be isolated from a partially fermented lactose solution. A further contribution by Willstätter and Bamann [1926, 2] showed that even yeasts rich in maltase will ferment without antecedent hydrolysis at p_H 5, at which maltase is practically inactive whilst fermentation proceeds at an optimum rate.

The authors feel that this problem forms a part of the general question, whether the metabolism of high-molecular compounds follows exclusively those patterns presented by the action of non-specific laboratory reagents such as acids and alkalis. The following report includes unpublished observations on yeast M made by one of us in 1921 under the direction of Prof. R. Willstätter [Sobotka,

1922], and a series of experiments with a peculiar yeast which was very kindly supplied by Mr Leo Wallerstein.

Yeast M of the Berliner Institut für Gärungsgewerbe, recommended for the fermentation of molasses, is, according to German authors, a mixture of five strains of yeast, among them yeasts II and XII. This statement is hardly in agreement with the fact that yeasts II and XII have maltase values of 1·3 and 4–6, while no sample of yeast M ever contained more than 0·4 maltase units per g. Samples of yeast M of maltase values 0·1–0·05 fermented maltose almost as fast as glucose, except, under the chosen conditions, for a peculiar period of induction of about two hours. Such prolonged inductions have never been observed with live yeast, whilst they are familiar with dry yeast and yeast juice fermentations [Sobotka, 1924]. Contact of the yeast with maltose solution abolishes induction, but even a prolonged stay of the yeast in a maltose medium and frequent renewal of maltose failed to enhance the maltase content of the yeast, while its fermenting power for maltose reached that for glucose during this maltose treatment.

The possibility of maltase formation during maltose fermentation was excluded in the following experiment. A maceration juice prepared according to Lebedev [1912] from an air-dried brewer's yeast, rich in maltase, contained exactly the same amount of maltase as the fresh yeast from which it was derived. A maceration juice from dry yeast M corresponded in its poor maltase content with fresh yeast M, whilst it fermented maltose. This observation supports the assumption of direct fermentation, as it excludes regeneration of maltase during fermentation, which is inconceivable in the cell-free juice. It also corroborates the soundness of the maltase determination with ethyl acetate on fresh yeast, as any cell-bound enzyme, which might have escaped that determination, should have been liberated during maceration.

Proof that the "malto-zymase" is not only free from a maltase component but actually follows a different initial path from "gluco-zymase" was obtained with the yeast from the Wallerstein Laboratories. This strain is distinguished by its ability to live, to ferment and to propagate at the rather acid p_H 2·5. This yeast is free from maltase both at p_H 2·5 and at p_H 6·5, the optimum for the action of yeast-maltase. None the less, it speedily ferments maltose or malt extract media, and, in several experiments, the rate of maltose fermentation exceeded that of glucose fermentation. These observations can be interpreted only on the assumption of direct fermentation of maltose.

Experimental.

Determination of maltase value and maltase units. Willstätter *et al.* [1924] defined as Maltase Unit (M.U.) that amount of enzyme which hydrolyses 2·5 g. maltose hydrate in 50 ml. of a phosphate buffer solution of p_H 6·8 at 30° to the extent of 50 % within one minute. A yeast has the Maltase Value (M.V.) = 1 if it contains 1 M.U. in 1 kg. dry substance.

The M.V. is usually estimated in the following manner. After the dry weight of the yeast has been determined, an amount equivalent to 2·5 g. dry yeast, usually about 10 g. fresh yeast, is liquefied with 1 ml. ethyl acetate, following the procedure of Willstätter and Steibelt [1920]. After cautious neutralisation with a suitable amount of $N/10$ ammonia, the solution is made up to 50 ml.; 40 ml. of this are added to 50 ml. of a 10 % maltose hydrate solution *plus* 5 ml. of $M/3$ phosphate buffer (equal parts of $M/3$ KH_2PO_4 and Na_2HPO_4) and made up to 100 ml. Samples of 25 ml. are withdrawn at appropriate intervals, *e.g.* 10, 20 and 40 minutes, and the action of the enzyme is interrupted by running the sample

into 5 ml. of a 2*N* Na_2CO_3 solution, which at the same time catalyses complete mutarotation of the products of hydrolysis [Hudson, 1908]. The samples are clarified by filtration, if necessary with addition of charcoal. The rotation in a 2 dm. tube of the clarified solution will be found to be +10·80° for a sample taken immediately after addition of the enzyme, and +4·40° after complete hydrolysis. Thus, percentage hydrolysis can be determined and the time for 50 % hydrolysis can be interpolated by means of the typical reaction curves [see Bamann, 1929].

If 50 % hydrolysis is accomplished, *e.g.* in 70 minutes, 50 ml. of the reaction mixture, equivalent to 1 g. dry yeast, contain 1/70 M.U. Thus, the M.V. of this yeast would be 1000/70 = 14·3.

Fermentation of sugars can be measured in a similar way. 20 ml. of a 5 % sugar solution, saturated with carbon dioxide, are fermented with fresh yeast equivalent to 0·2 g. dry yeast, and the carbon dioxide developed is measured, preferably over mercury. The amount of carbon dioxide developed under 760 mm. Hg pressure and 30° is 282 ml. The time within which half this amount is liberated can be correlated with the time of half hydrolysis under the conditions of the maltase determination, which are all identical except that the double amount of yeast is employed. The values for half fermentation of maltose, given in Table I, were obtained by this procedure.

Table I. *Fermentation and hydrolysis of maltose by various yeasts.*

Yeast	Time of half fermentation mins.	Time of half hydrolysis mins.	Maltase value
Munich brewer's	80	60	33·3
Danish distiller's	255	320	6·2
XII, distiller's	205	300–760	6·6–2·6
New York baker's	600	400–900	5·0–2·2
New York ale	—	900	2·2
Rhine wine	—	500–640	4·0–3·1
New York lager	460	4,000	0·5
Munich brewer's, acid-treated	170	4,000	0·5
Frankenthal distiller's	240	50,000	0·04
Vienna-Stadlau distiller's	1200	8,000–25,000	0·25–0·08
M, distiller's	480	5,200–16,000	0·38–0·12
A, grown at p_H 2·5	800	40,000	0·05

Induction period. Yeasts poor or lacking in maltase ferment maltose, as a rule, only after a delay of 10 minutes to 3 hours under the above conditions. After this induction period which lasts from 2 to 3 hours with yeast M, the development of carbon dioxide soon reaches the linear course typical for alcoholic fermentation. If this period of induction is subtracted from the time of half

Table II. *Fermentation of glucose and maltose by yeast M.*

	Time for half fermentation			Corrected time for half fermentation mins.
	Glucose mins.	Maltose mins.	Induction mins.	
Original sample (1. vii. 21)	200	380	120	260
After glucose passage	270	390	110	280
After 1 maltose passage	250	280	none	—
After 2 maltose passages	240	255	none	—
Same, 5 times washed	205	335	none	—
Original sample (22. vii. 21)	—	390	85	305
After maltose passage	210	220	none	—

fermentation in the case of yeast M, it is found that the maltose fermentation, once it has started, proceeds at the same rate as glucose fermentation. If fresh yeast M is carried through one or two passages in diluted malt extract, the induction period disappears. Maltase determinations in yeast M, after maltose treatment had eliminated the induction period, still yielded values of 9000 and 17,000 minutes as time for half hydrolysis. Analysis of reaction mixtures at the end of the induction period proved the absence of glucose.

Maltase determination in maceration extract. Maceration extract [Lebedev, 1912] was prepared in the following manner. Yeast was dried in a 2 mm. layer between 30° and 40°. One part of dry yeast was incubated at 30° for 4 hours with three parts of water. Three ml. of clear juice, isolated by centrifuging, corresponded to 1 g. dry yeast. Three or six ml. of juice were used in a total volume of 100 ml. buffered solution containing 5 % maltose; fermentation was effectively prevented by the addition of 5 ml. ethyl acetate. The figures given in Table III show that fresh maceration extract from brewer's yeast contains an

Table III. *Maltase determinations in fresh yeasts and maceration extracts.*

	$p_H = 6{\cdot}8$, $t = 30°$				
	Dry yeast equivalent g./100 ml.	Time min.	Decrease in rotation ($l = 2$ dm.)	% hydrolysis	Time of half hydrolysis, calc. for 1 g. dry yeast in 100 ml. min.
Löwenbräu yeast	2·0	40	2·80°	43·8	56
	—	80	3·72	58·2	56
Löwenbräu, maceration extract, 10 ml.	3·33	40	4·14	64·7	63
Same, after 24 hours, 6 ml.	2·0	30	1·74	27·2	235
	—	65	2·52	39·4	235
	—	120	3·25	50·8	330
Yeast M	2·0	160	0·25	3·9	7000
Same	2·0	450	1·00	15·6	5200
Yeast M, maceration extract, 6 ml.	2·0	1200	1·92	30·0	7800
Same, after 24 hours, 6 ml.	2·0	91	0·10	1·6	14,000
	—	132	0·18	2·9	13,500

amount of maltase identical with that found in the fresh yeast. It deteriorates like other enzymes in maceration extract rather rapidly. On the other hand, a fresh extract made from dried yeast M contained minute amounts of maltase only, of the same order of magnitude as the fresh yeast.

Yeast A. The yeast obtained from Mr Wallerstein, briefly designated yeast A, grows in a 10 % solution of malt extract the p_H of which is brought to 2·5–2·8 by addition of 0·8 volume % concentrated sulphuric acid. No maltase was found in this yeast when it was tested either at p_H 6·5 or at p_H 2·5. However, it fermented both maltose and glucose.

Fermentation was carried out in a Barcroft-Warburg apparatus, both in oxygen and in nitrogen, and the rate of carbon dioxide development was measured. This varied between 100 and 250 mm.^{3}/30 minutes, when an amount of fresh yeast equivalent to 4 mg. dry yeast was allowed to act on 50 mg. sugar in a total volume of 2 ml. $M/20$ primary phosphate solution. A comparison between maltose and glucose fermentation showed no significant difference between the two sugars. However, with one batch, the fermentation of maltose

was definitely faster than that of glucose. 208 mm.^{3} carbon dioxide were developed from maltose in thirty minutes as compared with 161 mm.^{3} from glucose under anaerobic conditions; under aerobic conditions the figures were 129 mm.^{3} for maltose and 73 mm.^{3} for glucose.

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CII. THE RELATION OF INTERMEDIARY METABOLIC PRODUCTS TO ARGINASE ACTIVATION.

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The catheptic enzymes, which are concerned with proteolysis in animal tissue, undergo activation when treated with certain substances, such as HCN or H_2S. These characteristic activation phenomena became of increased physiological interest with the discovery of the occurrence in tissues of natural activators, as for example, zookinase [Waldschmidt-Leitz *et al.*, 1930], which has been shown by Waldschmidt-Leitz and Purr [1931] to be identical with reduced glutathione. The same activator has been described by Grassmann *et al.* [1931] for the plant proteases (papain, bromelin and yeast-proteinase). In later experiments it was established [Waldschmidt-Leitz, Scharikova and Schäffner, 1933] that arginase, another enzyme concerned with intermediary protein metabolism, is also activated directly by metal complexes of SH compounds, for example, by reduced glutathione and ferrous or cuprous ions.

The interpretation of these activations as a specific function of the sulphydryl group [Waldschmidt-Leitz, Weil and Purr, 1933] cannot be considered as valid, for we have found that other types of substances, as for example, ascorbic acid-$Fe^{=}$, methylglyoxal-$Fe^{=}$, and alloxan-$Fe^{=}$ exercise the same activating effect on arginase as is obtained with the sulphydryl-$Fe^{=}$ system (see Tables V, II and I). These newly demonstrated activators however possess in common the property of forming reversible redox systems. The hypothesis of a connection between arginase activation and the oxidation-reduction potential, for the establishment of which oxygen [Waldschmidt-Leitz, McDonald *et al.*, 1933] or hydrogen [Edlbacher *et al.*, 1933] is necessary, is in harmony with the experimental findings and accounts for the activations observed with the above-mentioned compounds.

Investigations reported by various writers during the past year have shown that in general these oxidations and reductions are regulated by reversible redox systems, which in the presence of available oxygen or hydrogen show variable potentials. Of significance to the investigation is the fact that the potential is determined not by the total amount of the system present but only by the ratio of the reduced to the oxidised phase. The total amount acts to a certain extent however as a buffer. With a constant ratio of reduced form to oxidised form, it is apparent that the redox potentials will vary also with the p_H. With increasing alkalinity they become more negative (more intense), and with increasing acidity, less negative (less intense). The particular physiological significance of such biological oxidations and reductions is dependent on the one hand on the assumption of a capacity to absorb such reduction potential (specificity) and on the other hand on the intensity of such potential. It will

thus be necessary, in enzyme activations, to distinguish between a specificity factor, and an intensity (activation) factor, which latter is known to be influenced by p_H and is responsible for the attainment of the full activity of enzymes.

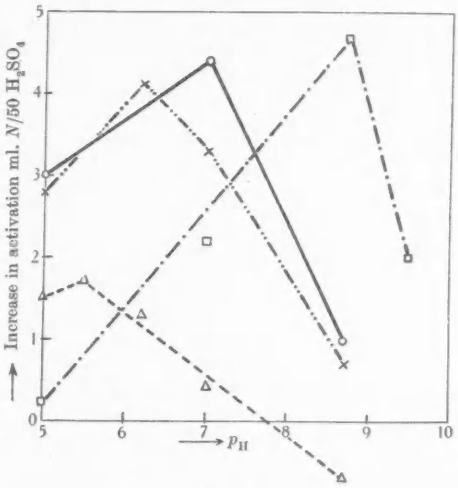

Fig. 1.

Cysteine-iron ($Fe^{=}$). — · — Cysteine.
- - - - Bivalent iron. —··— Ascorbic acid-iron ($Fe^{=}$).

From the curves (Fig. 1) which represent the experiments with $Fe^{=}$, cysteine, cysteine-$Fe^{=}$ and ascorbic acid-$Fe^{=}$, it is clear that the optimum development of the intensity factors of arginase, which is the development of the full activity, is dependent, as previously mentioned, on the p_H. Thus one obtains, by shifting the p_H to the alkaline side, the same activation intensity optimum as is obtained at neutral reaction by the addition of bivalent iron. While cysteine in acid medium is indifferent or even inhibiting, bivalent iron shows, to a less extent, the opposite picture. Bivalent iron therefore acts in a certain sense as a p_H buffer with respect to the development of the specific intensity potential which is required by the arginase, and it may also act in the same way in the organism. With ascorbic acid-$Fe^{=}$, the activation intensity optimum is shifted still more to the acid side than is the case with cysteine-$Fe^{=}$. In agreement with these results, Hill and Michaelis [1933] have found that the potential developed in the system alloxantin-$Fe^{=}$ is also dependent on the p_H, the bivalent iron apparently being a determining factor.

In a previous communication [Purr, 1933] an activation of arginase by ascorbic acid-$Fe^{=}$ was reported. In connection with this finding, the question was raised as to what extent other intermediary metabolic products exert a regulating effect on intracellular enzymic syntheses or hydrolyses. The experiments here reported, which have been carried through from this point of view, have shown that the activation mechanism of this enzyme is probably connected with a specific oxidation-reduction potential, and is not due simply to a specific effect of the SH group.

Experimental.

The activation of arginase.

The organs studied were rat liver and transplantable rat sarcoma, Philadelphia # 1[1]. Immediately after extirpation these tissues were cut up with scissors and frozen by dipping into liquid nitrogen, then pulverised by crushing with a hammer. This finely crushed material was suspended as follows: cancer (Philadelphia # 1 rat sarcoma) in 90 % glycerol (1: 10); rat liver in 90 % glycerol (1: 10); glycerol extract from liver acetone-ether dry preparation

Table I. *The effect of alloxan, allantoin, alloxantin and uric acid.*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or 20·0 mg. alloxan, 20·0 mg. allantoin, 20·0 mg. alloxantin, 20·0 mg. uric acid, respectively. p_H 7.

	Enzyme		
Activator system	Liver acetone-ether dry preparation (ml. 0·02 *N* H_2SO_4)	Rat liver suspension (ml. 0·02 *N* H_2SO_4)	Cancer suspension (Philadelphia # 1 rat sarcoma) (ml. 0·02 *N* H_2SO_4)
Initial activity	6·8	7·0	2·3
$Fe^{=}$	14·6	6·4	3·3
Cysteine	7·1	8·1	5·8
Cysteine-$Fe^{=}$	20·4	15·2	7·9
Alloxan	8·4	—	3·2
Alloxan-$Fe^{=}$	19·3	—	8·0
Allantoin	8·6	—	—
Allantoin-$Fe^{=}$	15·4	—	—
Alloxantin	—	7·9	—
Alloxantin-$Fe^{=}$	—	9·6	—
Uric acid	6·2	—	—
Uric acid-$Fe^{=}$	11·6	—	—

Table II. *The effect of methylglyoxal, pyruvic acid and* d*-lactic acid.*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or 16·0 mg. methylglyoxal, 20·0 mg. pyruvic acid, 20·0 mg. *d*-lactic acid, respectively. p_H 7.

	Enzyme		
		Cancer suspension (Philadelphia # 1 rat sarcoma) (ml. 0·02 *N* H_2SO_4)	
Activator system	Rat liver suspension (ml. 0·02 *N* H_2SO_4)	No. 1	No. 2
Initial activity	7·0	7·7	3·4
$Fe^{=}$	6·4	7·6	4·7
Cysteine	8·1	10·8	4·3
Cysteine-$Fe^{=}$	15·2	10·8	8·2
Methylglyoxal	—	2·0	4·0
Methylglyoxal-$Fe^{=}$	—	18·6	7·7
Pyruvic acid	7·1	—	—
Pyruvic acid-$Fe^{=}$	6·3	—	—
d-Lactic acid	6·2	—	—
d-Lactic acid-$Fe^{=}$	6·7	—	—

[1] The pathological description of this transplantable rat sarcoma, Philadelphia # 1, can be found in the article "Ueber die Enzyme in Tumoren," by Waldschmidt-Leitz, McDonald *et al.* [1933].

(1 : 10). The amounts used were: 5·0 ml. cancer suspension; 0·25 ml. liver suspension; 0·5 ml. extract of acetone-ether dry preparation. For activation 2·0 ml. neutral cysteine-HCl solution (containing 20 mg. cysteine-HCl) or 0·5 ml. 0·1 *N* $FeSO_4$, respectively, were added to the enzyme and allowed to stand 1 hour at p_H 7 at 30°. 10 ml. of 1 % arginine carbonate and 5·0 ml. 0·1 *M* glycine buffer of p_H 9·5 were then added and the reaction mixtures allowed to stand 60 minutes at 30°.

Results are shown in ml. 0·02 *N* H_2SO_4, as is customary.

Table III. *The effect of thiolacetic acid.*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or 20·0 mg. thiolacetic acid, respectively. p_H 7.

Activator system	Enzyme. Cancer suspension (Philadelphia # 1 rat sarcoma) (ml. 0·02 *N* H_2SO_4)
Initial activity	6·0
$Fe^{=}$	10·5
Cysteine	8·1
Cysteine-$Fe^{=}$	14·5
Thiolacetic acid	6·8
Thiolacetic acid-$Fe^{=}$	12·5

Table IV. *The effect of bivalent iron in complex combination.*

(Potassium ferrocyanide, $Fe^{=}$-dimethylglyoxime and αα′-dipyridyl[1].)

The enzyme was activated by 20·0 mg. potassium ferrocyanide; 20·0 mg. dimethylglyoxime; 5 mg. dipyridyl; (A) after 1 hour's standing with dipyridyl, $FeSO_4$ was added and the mixture allowed to stand another hour, p_H 7; (B) the same with cysteine instead of $FeSO_4$; (C) the same with cysteine-$Fe^{=}$; (D) after 1 hour's standing with dipyridyl, and another after addition of $FeSO_4$, cysteine-$Fe^{=}$ was added and the mixture allowed to stand an additional hour.

	Enzyme	
Activator system	Liver acetone-ether dry preparation (ml. 0·02 *N* H_2SO_4)	Rat liver suspension (ml. 0·02 *N* H_2SO_4)
Initial activity	6·8	7·0
$Fe^{=}$	14·6	6·4
Cysteine	7·1	8·1
Cysteine-$Fe^{=}$	20·4	15·2
Potassium ferrocyanide	3·6	—
Dimethylglyoxime	3·3	—
Dimethylglyoxime-$Fe^{=}$	8·3	—
αα′-Dipyridyl	—	6·2
(A)	—	1·0
(B)	—	5·0
(C)	—	14·4
(D)	—	3·0

From these figures it is evident that ferrous compounds in their rôle as activator for arginase are not interchangeable with bivalent iron in complex combination. It is also shown that ferrodipyridyl (A) inhibits irreversibly the action of arginase.

[1] The authors wish to thank Dr L. Michaelis, of the Rockefeller Foundation for Medical Research, for his kindness in supplying the αα′-dipyridyl.

Table V. *The effect of* p_H *(specificity of activation).*

The enzyme was activated by 0·5 ml. 0·1 *N* $FeSO_4$ or by 20 mg. cysteine-HCl or 16 mg. ascorbic acid ("chinoin") respectively, and allowed to stand 1 hour at p_H 5·0, 5·5, 6·2, 7·0, 8·7, 9·5 at 30° (the p_H was controlled). The activation of arginase was determined as usual at p_H 9·5. (Enzyme influence in ml. 0·02 *N* H_2SO_4.)

	Enzyme. Rat liver suspension					
p_H	5·0	5·5	6·2	7·0	8·7	9·5
Activator system						
Initial activity	—	—	—	4·7	—	—
$Fe^{=}$	6·2	6·4	6·0	5·1	4·0	—
Cysteine	4·5	—	—	6·9	9·4	6·7
Cysteine-$Fe^{=}$	7·7	—	—	9·1	5·7	—
Ascorbic acid-$Fe^{=}$	7·5	—	8·8	8·0	5·4	—
Increase in activation						
$Fe^{=}$	1·5	1·7	1·3	0·4	−0·7	—
Cysteine	0·2	—	—	2·2	4·7	2·0
Cysteine-$Fe^{=}$	3·0	—	—	4·4	1·0	—
Ascorbic acid-$Fe^{=}$	2·8	—	4·1	3·3	0·7	—

SUMMARY.

It has been shown that various intermediate products of metabolism, in combination with ferrous iron, are able to activate arginase. The activation mechanism of this enzyme is probably connected with a specific oxidation-reduction potential and is not due simply to a specific effect of the SH group.

We wish to express our thanks to Doctor Ellice McDonald, Director, for his interest and support throughout this work.

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CIII. PYRUVIC ACID IN THE DISSIMILATION OF GLUCOSE BY THE PROPIONIC ACID BACTERIA[1].

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(*Received February 22nd, 1934.*)

ALTHOUGH Van Niel [1928] postulated that pyruvic acid is formed during the dissimilation of glucose by the propionic acid bacteria, the keto-acid has never been identified as an intermediate product. Van Niel assumed that pyruvic acid hydrate was formed and subsequently dehydrogenated to yield acetic acid, carbon dioxide and two atoms of hydrogen, the latter serving to reduce suitable hydrogen acceptors such as methylglyoxal to propionic acid.

$$CH_3.C(OH)_2.COOH \rightarrow CH_3COOH + CO_2 + 2H \qquad \ldots\ldots(1).$$

This assumption was necessitated by the failure to demonstrate the conversion of acetaldehyde into acetic acid or the presence of acetaldehyde itself during the fermentation of glucose by the propionic acid bacteria, although acetaldehyde would be a normal product of the decarboxylation of pyruvic acid.

By assuming a dehydrogenation of pyruvic acid hydrate, the absence of acetaldehyde is accounted for.

Van Niel based his conclusion of the presence of pyruvic acid largely upon its ease of fermentation by the propionic acid bacteria with formation of propionic acid, acetic acid and CO_2 in substantially theoretically correct proportions.

Actual proof of the intermediate formation of pyruvic acid from glucose by propionic acid bacteria would evidently lend considerable support to Van Niel's theory of dissimilation of glucose by these organisms.

In the present communication we offer such proof in the form of the isolation and identification of pyruvic acid in the fermentation of glucose by *Propionibacterium arabinosum*.

EXPERIMENTAL.

The culture of *Propionibacterium arabinosum* used in this study was kindly furnished by Dr E. R. Hitchner of the University of Maine and was described in detail by Werkman and Brown [1933].

The fermentations were carried out in a medium of Bacto yeast extract 0·5 %, glucose 2·0 %, and calcium carbonate 2 %. Either sodium bisulphite (0·5 %) or calcium sulphite (1·0 %) was included in the medium as a fixative. Constituents of the medium were sterilised separately and mixed. A number of 500 ml. flasks each containing 300 ml. of medium were prepared and the fermentations carried out anaerobically by bubbling oxygen-free nitrogen through the medium. The inoculum was a five-day culture of the organism in glucose yeast extract broth added in quantity equivalent to 5 % by volume of

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the medium. Incubation was at 30°. Growth was luxuriant and the glucose was completely utilised by the tenth day of fermentation. The cultures were proven pure by microscopic examination and colony formation in shake tubes and on agar plates.

Both the sodium bisulphite and the calcium sulphite were effective in fixing pyruvic acid. The results were substantially similar with the two fixatives. The following experiment is typical. Tests for acetaldehyde following distillation of the medium according to the method of Peterson and Fred [1920] were negative. Upon addition of 2 : 4-dinitrophenylhydrazine to the medium an abundant precipitate was obtained which was dissolved in sodium carbonate solution and reprecipitated by acidification. After purification of the 2 : 4-dinitrophenylhydrazine precipitate, the melting-point was 212·0°; a mixture with authentic pyruvic acid 2 : 4-dinitrophenylhydrazone (M.P. 213° [*cf.* Allen, 1930]) melted at 212°.

A *p*-nitrophenylhydrazone was likewise prepared from the fermentation solution; it had M.P. 218·5° alone and 218·5° when mixed with authentic pyruvic acid *p*-nitrophenylhydrazone (M.P. 219°).

Discussion.

This identification of pyruvic acid in the fermentation of glucose by *Propionibacterium arabinosum* supports the scheme of glucose dissimilation proposed by Van Niel. The facts that pyruvic acid is fixed during the fermentation and that no acetaldehyde can be detected do not indicate a decarboxylation of pyruvic acid. Carboxylase is reported by Neuberg and Reinfurth [1920] to act upon the bisulphite addition compound of pyruvic acid to form the acetaldehyde bisulphite compound, so that in the presence of carboxylase, fixation of pyruvic acid by sulphite should not prevent simultaneous formation of acetaldehyde. Isolation of pyruvic acid was successful in the present case, apparently owing to the inability of *P. arabinosum* to decarboxylate the pyruvic acid bisulphite compound which is not itself subject to hydration and subsequent dehydrogenation as is pyruvic acid.

The reason for the reported failure of Virtanen [1923] to secure growth of the propionic acid bacteria in the presence of sulphite is not apparent.

The authors found no account in the literature of a similar isolation of pyruvic acid from a sulphite-containing culture of actively proliferating bacteria, although Cook [1930] identified pyruvic acid in a fermentation by resting *Bact. coli* using $NaHSO_3$ as a fixative. This fact may well be the result of the unusual mechanism of the dissimilation of glucose brought about by the propionic acid bacteria in which acetaldehyde does not occur as an intermediate product. This mode of formation of acetic acid and carbon dioxide from pyruvic acid explains their formation in the ratio of 1 : 1, a ratio that the authors have not been able to alter.

With regard to the mode of formation of the pyruvic acid from glucose, the evidence is fragmentary; possibly pyruvic acid arises from the dehydrogenation of methylglyoxal hydrate or lactic acid. The latter substance probably occurs as an intermediate only under certain conditions when it is formed as a stabilisation product. Its occurrence has been frequent in large fermentations conducted in this laboratory.

SUMMARY.

Pyruvic acid has been isolated by fixation with sulphite in the fermentation of glucose by *Propionibacterium arabinosum*.

Acetaldehyde has not been detected in these experiments by the sulphite fixation method.

The scheme proposed by Van Niel for the intermediate breakdown of pyruvic acid is shown to be in agreement with experimental results.

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CIV. STUDIES ON THE KINETICS OF HAEMOLYTIC SYSTEMS.

V. A FURTHER INVESTIGATION OF THE INHIBITION PRODUCED BY PLASMA AND SERUM.

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(*Received February 23rd, 1934.*)

If a quantity of plasma or serum is added to a haemolytic system containing a quantity c_1 of a lysin such as saponin or one of the bile salts, the time t_2 required for complete lysis is longer than the time t_1 which would be required if the quantity c_1 were to act in the absence of the added plasma or serum. It seems, indeed, that a quantity of lysin Δ is rendered inactive by the plasma, the remainder of the lysin, c_2, producing lysis in the time $t_2 > t_1$. The problem is to find a simple and satisfactory relation between the variables.

One of us attempted to do this in 1923 [Ponder, 1923], and influenced by the idea that plasma-proteins might "adsorb" some of the lysin, sought a relation between Δ, the amount "adsorbed," and c_2, the amount left free. These two quantities were found to be related by an expression not unlike the adsorption isotherm,

$$\Delta = \alpha c_2^{1/\mu} \qquad \text{.....(1),}$$

where α and μ are constant, provided that any one amount of plasma M is used as the inhibitor. Plotting $\log \Delta$ against $\log c_2$ thus resulted in a straight line with a slope of $1/\mu$, but when the value of M was varied, μ was found to vary from about 3·5 when M was 0·04 ml. to about 0·035 when M was 0·00125 ml. The straight lines corresponding to various values of M accordingly cut one another: "an impossible state of affairs" [Ponder, 1925].

This difficulty was in part overcome in 1925 by the observation that the curves obtained by plotting Δ against c_2 do not pass through the origin, but cut the c_2 axis at different levels. To allow for this, expression (1) was re-written

$$\Delta = \alpha\,(c_2 - K)^{1/\mu} \qquad \text{.....(2),}$$

where K is the intercept on the c_2-axis just referred to. The plotting of $\log \Delta$ against $\log (c_2 - K)$ now resulted in a series of parallel straight lines with $\mu = 1{\cdot}75$: this is a much simpler state of affairs, offset, however, by the fact that no good explanation was forthcoming either for the existence of K or for the fact that K was found to vary with M in a quite inexplicable way [Ponder, 1925].

In view of this new difficulty, and in view of the possibility that the premises underlying the analysis might be altogether wrong, a very rigorous test was applied [Ponder, 1932]. If the effect of the added inhibitor is to render lysin inert, and not to interfere with the haemolytic reaction in any other way, we must have the following relations fulfilled,

$$\left.\begin{aligned} \Delta_1/\Delta_2 &= 1 \\ \Delta_1 &= c_1 - 1{\cdot}2c_2 \\ \text{and} \quad \Delta_2 &= c_1 - 1{\cdot}2c_3 \end{aligned}\right\} \qquad \text{.....(3),}$$

where c_1 is the initial amount of lysin in the system containing the plasma, c_3 the quantity apparently left free after some of the lysin Δ_2 has been rendered inert as judged by the time required for complete haemolysis, and c_2 the quantity left free after some of the lysin Δ_1 has been rendered inert, as judged by the time required to produce 50 % (or any other percentage) haemolysis. It was found that this condition was very exactly fulfilled, and so the hypothesis that the plasma inactivated lysin without interfering with the other properties of the system was supported by evidence not easy to set aside.

The present situation is therefore very unsatisfactory. The assumption that the plasma reacts with the lysin so as to render some of it inert is apparently sound, but the relation between Δ and c_2 cannot be left as it stands, for either we have to explain the variation of μ with M, or we have to introduce a third constant K, itself a function of M, and devoid of physical meaning. The following investigations will therefore be divided into three parts.

1. Remembering that all previous analyses have been based on data obtained by plotting "short" time-dilution curves, we shall show that essentially the same relations result from analysing data based on the more accurate "long" time-dilution curves [see Ponder and Yeager, 1930].

2. We shall show that the order of the fundamental reaction between cell and lysin is not changed as a result of the presence of the inhibitor, *i.e.* we shall confirm by independent means the result of the test contained in expression (3).

3. Abandoning the idea of an "adsorption," we shall seek a relation between Δ and c_1. This will be found to involve the constants α, μ and K, but the last mentioned will be assigned an obvious physical meaning, and it will be shown that μ is independent of the amount of inhibitor used. In this way the problem will be solved in terms of a single expression, which, although empirical, has a real physical significance. It will further be shown that we are now able to predict, in a qualitative sense at least, the changes which are observed in the amount of inhibition when the number of cells in the systems is varied.

Table I.

	δ_1	c_1	t	δ_2	c_2	Δ
Saponin *plus* 0·02 ml. serum	30,000	66·7	4·0	62,000	32·4	34·3
	35,000	57·1	10·2	75,000	26·6	30·4
	40,000	50·0	30·0	87,000	23·0	27·0
	45,000	44·5	90·0	99,000	20·2	24·3
Saponin *plus* 0·16 ml. serum	30,000	66·7	2·5	54,000	36·7	30·0
	40,000	50·0	11·0	78,000	25·6	24·4
	45,000	44·5	26·0	86,000	23·2	21·3
	50,000	40·0	120·0	101,000	19·8	20·2
Saponin *plus* 0·01 ml. serum	35,000	57·1	3·5	56,000	35·7	21·4
	40,000	50·0	5·1	66,000	30·3	19·7
	45,000	44·5	9·0	76,000	26·3	18·2
	50,000	40·0	16·0	84,000	23·8	16·2
	55,000	36·3	37·0	89,000	22·4	13·9
Saponin *plus* 0·006 ml. serum	40,000	50·0	2·6	55,000	36·3	13·7
	50,000	40·0	6·1	72,000	27·8	12·2
	55,000	36·3	11·0	79,000	25·3	10·7
	60,000	33·3	26·0	86,000	23·2	10·1
	65,000	30·3	48·0	91,000	22·0	8·0
Saponin *plus* 0·004 ml. serum	45,000	44·5	3·5	56,000	35·7	8·8
	50,000	40·0	4·5	63,000	31·8	8·2
	55,000	36·3	7·0	70,000	28·6	7·7
	60,000	33·3	10·5	77,000	26·0	7·3
	65,000	30·3	40·4	89,500	23·7	6·6

Data for "long" time-dilution curves.

These are presented in Table I, and arranged in exactly the same way as in the Table on p. 486 of the paper which contains the results obtained from "short" time-dilution curves [Ponder, 1925]. All the figures refer to time-dilution curves for saponin and a standard suspension of human red cells at 30°. Values of c_1, c_2 and Δ are in γ.

The effect of added plasma on the order of the fundamental reaction.

The time-dilution curves for systems containing various quantities of plasma (or serum) can be individually subjected to analysis for the values of the constants in the equation which describes them,

$$kt = \frac{p}{p-1} \cdot \left\{ c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}} \right\} \qquad \ldots\ldots(4),$$

and, using the data given *in extenso* above together with those for some intermediate curves, we get the values shown in Table II.

Table II.

Serum, ml.	$n\ (=1/p)$	k	x	$R\ (=x_1/x_2)$
0·0	2·6	0·00213	19·0	1·00
0·02	2·6	0·000557	40·0	2·11
0·016	2·6	0·000813	36·4	1·92
0·010	2·6	0·00104	30·8	1·62
0·008	2·6	0·00108	28·6	1·51
0·006	2·6	0·00147	26·7	1·41
0·004	2·6	0·00152	24·1	1·27
0·002	2·6–2·7	0·00174	23·0	1·21

Thus n is constant, irrespective of the quantity of inhibitor added, as required if the test in expression (3) is to apply. When the analysis of the curves is carried out in this way, k decreases as M increases, but this is only because the analysis in terms of expression (4) takes no account of the inhibitor rendering lysin inert. The decreasing value of k is really only another way of expressing the relation of Δ to c_1, as in expression (5).

The accuracy with which the various time-dilution curves are described by expression (4), with the above values of the constants inserted, is really quite surprising.

Results of plotting Δ against c_1.

Plotting Δ against c_2 results in exactly the same type of curve as in previous investigations, the curves corresponding to each value of M cutting the c_2-axis at various levels, and being described with a fair degree of accuracy by expression (2), which contains the arbitrary constant K, a function of M. Plotting Δ against c_1, on the other hand, results in a series of curves which cut the c_1-axis at a single point, so that $\Delta = f(c_1 - K)$, K now being a constant for all values of M. (This is clearly shown in Fig. 1, in which the two methods of plotting are compared.) To find a meaning for K, consider the steps of the analysis in detail. The time-dilution curve in the absence of inhibitor has an asymptote at $c_{1\infty}$, for which concentration $t = \infty$. Imagine a time-dilution curve for a system containing an indefinitely small amount of plasma: this curve will differ insensibly from the "standard" curve, and will have an asymptote $c_{2\infty}$, differing insensibly from $c_{1\infty}$. Thus when Δ tends to zero, c_2 will tend to c_1, and the curve obtained by plotting Δ against c_1 will cut the c_1-axis at a point $c_1 = c_{1\infty}$, $\Delta = 0$, and this will be the intercept K, equal to $c_{1\infty}$. The constant K is therefore equal to the asymptotic

concentration for the "standard" curve with which the time-dilution curve for the system containing plasma is compared, and expression (2) becomes

$$\Delta = \alpha (c_1 - c_{1\infty})^{1/\mu} \qquad \ldots\ldots(5).$$

Plotting $\log \Delta$ against $\log (c_1 - c_{1\infty})$ now gives a series of parallel straight lines for which $\mu = 2{\cdot}0$ (in this case) and for which α has the following values:

Serum, ml.	α
0·02	4·67
0·016	4·27
0·010	3·47
0·006	2·57
0·004	1·74

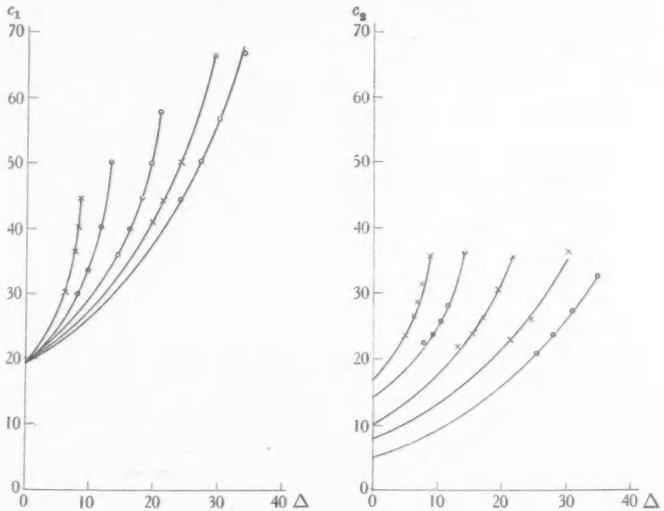

Fig. 1. To show the result of plotting Δ against c_1 and c_2 respectively. The five different curves correspond to five different quantities of serum.

The value of μ is therefore constant for all values of M, while the value of α decreases as M decreases. As has previously been remarked, it does not decrease linearly with M, and small amounts of inhibitor are relatively more effective than larger amounts.

Expression (5) accordingly has a comparatively simple physical meaning, and the only curious thing about it is that the quantity of lysin rendered inactive by the inhibitor varies as a power of the lysin initially present (or, for that matter, of the lysin left free). On the other hand, it is scarcely to be expected that a reaction between two such colloids as saponin and the serum-proteins would be expressible by anything better than an empirical formula.

The effect of varying the concentration of the cell suspension.

All previous investigations have been carried out with reference to suspensions of "standard" strength, but we shall now show that the phenomenon of inhibition by plasma and serum can be completely understood only if we consider what happens when the concentration of the cell suspension is varied.

Consider first what ought to happen if the foregoing account of the inhibition is adequate. Each one of a family of Δ_1, c_1 curves for different values of M, when obtained by using a suspension of a particular strength s_1, will be described by

$$\Delta = \alpha(c_1 - c_{1\infty\, s_1})^{1/\mu},$$

where $c_{1\infty\, s_1}$ is the asymptote of the standard curve. If a suspension of strength s_2 is used, and if the foregoing analysis takes account of all the facts, the members of the new family of Δ_1, c_1 curves should be described by

$$\Delta = \alpha(c_1 - c_{1\infty\, s_2})^{1/\mu},$$

where $c_{1\infty\, s_2}$ is the asymptote of the standard curve for the s_2 suspension. Thus each member of the second family ought to be the same as the corresponding member of the first family, except that the former is translated upwards or downwards by a distance equal to $(c_{1\infty\, s_1} - c_{1\infty\, s_2})$.

Experiment shows, however, that other differences are introduced by varying the strength of the suspension. For example, using a $2x$ standard and a $0{\cdot}5x$ standard suspension, we get the following figures:

	$\tilde{n}$	c_1	$c_1 - c_{1\infty}$	Δ
Saponin *plus* 0·16 ml. serum,	20,000	100·0	72·0	31·3
$2\times$S suspension	25,000	80·0	52·0	28·0
	30,000	66·7	38·7	24·8
	38,000	52·6	24·6	21·1
Saponin *plus* 0·16 ml. serum,	25,000	80·0	55·6	33·1
$0{\cdot}5\times$S suspension	30,000	66·7	42·3	30·0
	35,000	57·1	32·7	26·9
	37,000	54·1	29·7	26·3

Analysis of curves such as these shows (*a*) that the Δc_1 curves for the $0{\cdot}5x$ standard suspension make an intercept on the c_1-axis which is less than that made by the curves for the systems containing the $2x$ standard by the amount required by theory, but (*b*) that the value of α is not the same for the two families, although the value of μ is the same. This means that the quantity of lysin apparently inhibited by a particular quantity of serum or plasma depends on the number of cells present in the suspension used for obtaining the time-dilution curves, and that for the same values of c_1 more lysin appears to be inhibited when the strength of the suspension is small than when it is greater. At first this observation puzzled us a great deal, but it can be explained quite easily if we take into account certain other known properties of the systems under consideration. The explanation involves giving up the idea that there is complete irreversibility of the compound formed by the lysin and the plasma-proteins.

We know that when lysin is added to cells in concentration c_1, a quantity of lysin $= \zeta c_1$ is concentrated at the cell surfaces, thus leaving free a quantity $c_1(1-\zeta)$. So far as we know from rather limited observations, ζ is a constant. As lysis goes on, the quantity of "free" lysin decreases, but always in such a way that it is nearly a linear function of c_1 (see Ponder [1932] the section dealing with the "second addition effect"). In any system containing cells, lysin and inhibitor, we have therefore to distinguish between three quantities: the quantity of lysin "absorbed" on the cells, the quantity left free and the quantity inhibited as a result of its interaction with the serum or plasma-proteins. The relation of these quantities will be

	Absorbed	Free	Inhibited
Before cells added	0	c_2	c_3
After cells added	c_4	c_5	c_6

Remembering that $c_1=c_2+c_3=c_4+c_5+c_6$, and that $c_4=\zeta c_5$, and putting, for simplicity's sake, $c_3=\alpha c_2$, we get

	Absorbed	Free	Inhibited
Before cells added	0	$c_1/1+\alpha$	$\alpha c_1/1+\alpha$
After cells added	$\zeta c_1/1+\zeta+\alpha$	$c_1/1+\zeta+\alpha$	$\alpha c_1/1+\zeta+\alpha$

The addition of the cells should therefore result in the reduction of the amount of lysin inhibited from $\alpha c_1/1+\alpha$ to $\alpha c_1/1+\zeta+\alpha$. Clearly the greater ζ the greater the reduction; hence the greater the strength of the suspension used, the smaller will the quantity of lysin inhibited appear, for ζ increases as the number of cells increases. If $c_3=\alpha c_2^{1/\mu}$, no simple solution is possible, but it will be obvious that the effect will be in the same direction, and that the value of μ should be independent of the strength of the suspension. This is just what is found.

This theory appears entirely adequate, but it demands one rather curious condition in the haemolytic system in order that expression (3) be satisfied. When the cells are added to the system containing the plasma or serum, a redistribution of the quantities of lysin absorbed, free and bound to the inhibitor occurs in the way shown above. The course of the ensuing lysis, however, is exactly the same as that in a system containing no inhibitor, but having the lysin present in smaller initial concentration (see expression (3)). In fact, the redistribution having once been effected by the addition of the cells, the lysin bound to the inhibitor behaves as if it were permanently removed from the system. As haemolysis proceeds, the concentration of free lysin may fall, and probably does, but it is not continually replenished by the inhibitor giving up some of its bound lysin, as in a simple reversible system. We do not pretend fully to understand this, but it undoubtedly has a relation to a series of observations which will shortly be published elsewhere, and which shows that the kinetics of haemolysis require to be considered in relation to a sort of "internal" system rather than in relation to the system considered as a homogeneous whole, and that the properties of this "internal" system depend largely on the way in which the lysin is concentrated at the cell surfaces. In such systems, characterised by extreme heterogeneity, the laws for reactions in homogeneous systems certainly do not apply, and we require to seek information regarding their properties by experiment rather than by applying existing theory.

Summary.

This paper is concerned with a re-investigation of the problem of the inhibition of a simple lysin by plasma or serum, using better methods of experiment and analysis than have been used hitherto. The previous conclusion that it is permissible to treat the inhibition as a reaction between lysin and plasma-protein, by means of which the lysin is removed from the system, is confirmed. It is also shown that the quantity of lysin so removed depends on the number of cells present in the system, and this observation is shown to have a simple explanation.

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CV. THE EFFECT OF DESICCATED THYROID, IRRADIATED ERGOSTEROL AND AMMONIUM CHLORIDE ON THE EXCRETION OF CALCIUM IN RATS.

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(*Received February 12th, 1934.*)

Parhon [1912] observed a marked loss of calcium in rabbits which received lethal doses of thyroid substance by mouth. Aub *et al.* [1929] reported that patients with hyperthyroidism showed an excretion of calcium much greater than that observed on similar diets in hyperparathyroidism or after the administration of acids; he pointed out that the high calcium excretion of hyperthyroidism was unusual in that it occurred chiefly by the intestinal route. Parhon *et al.* [1932] found that demineralisation of the skeleton occurred in dogs during thyroid feeding and was not increased by simultaneous administration of parathyroid extracts. Tibbetts *et al.* [1932] announced that the high faecal excretion of calcium in clinical hyperthyroidism was not influenced by administration of irradiated ergosterol; Watchorn [1930], Pugsley [1932] and others have found however that irradiated ergosterol tends generally to decrease the faecal and increase the urinary excretion of calcium. The present paper reports a study of the effect of desiccated thyroid on the calcium excretion of rats and the influence of irradiated ergosterol[1] thereon, while some experiments on the effect of ammonium chloride acidosis are included for comparison.

Methods.

The experiments were similar in general plan and in the analytical methods used to those described by Pugsley [1932] in a study of the effects of parathyroid extracts and of irradiated ergosterol on the calcium and phosphorus metabolism of rats. Adult male and female rats of Wistar strain, weighing approximately 240 g., were used. The diet, which contained 0·056 % calcium, was given as a constant weighed ration daily, and was not increased during thyroid treatment in spite of the greater appetite of the animals; the weight of unconsumed food was also recorded. Metabolic rates were found by determination of oxygen consumption in a closed-circuit apparatus, similar to that described by Benedict and Macleod [1929], save that the fall of the oxygen reservoir-spirometer is traced on a rotating smoked drum, the tracing serving also as a record of activity. The metabolic rates are expressed as the percentage increase in oxygen consumption over the oxygen consumption of the individual animals determined in the pre-experimental period.

The desiccated thyroid was supplied by Messrs Ingram and Bell, and was found to contain 0·141 % calcium; it was thoroughly mixed with the food each day, so that each rat received 2 grains daily if, as was almost invariably the case, the entire ration was consumed. Irradiated ergosterol (Mead, Johnson & Co.)

[1] The authors are indebted to Mead, Johnson & Co. for the ergosterol used in these experiments.

was suitably diluted with olive oil and given from a pipette in daily doses of 0·1 ml. Ammonium chloride was given daily at the time of feeding by stomach tube, subcutaneous injection at the dosage required having proved highly toxic.

Results.

The effect of administering desiccated thyroid (2 grains daily) is shown in Fig. 1, which is a composite graph representing the average of six similarly

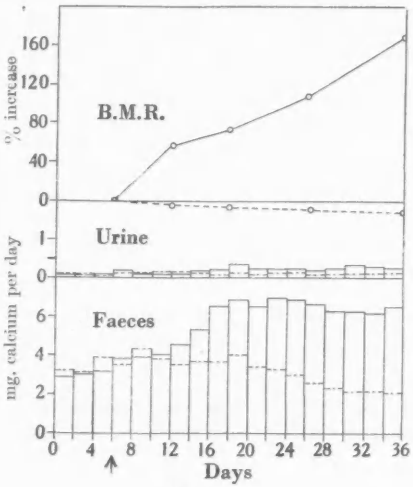

Fig. 1.

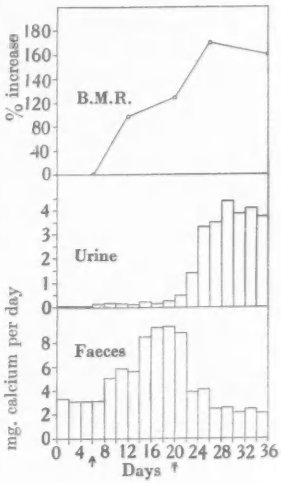

Fig. 2.

Fig. 1. Effect of administering 2 grains desiccated thyroid daily on calcium excretion (average of 6 rats); the dotted line shows the calcium excretion of controls.

Fig. 2. Effect of administering 20,000 units of irradiated ergosterol daily upon the increased calcium excretion induced by thyroid feeding (average of 6 rats). The first arrow indicates the commencement of thyroid treatment, the second the addition of irradiated ergosterol.

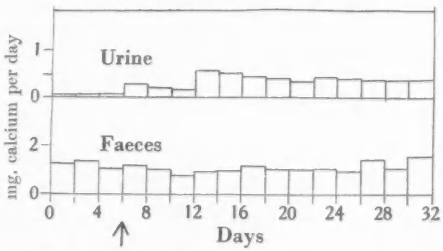

Fig. 3. Effect of administering 125 and (later) 250 mg. ammonium chloride daily on the calcium excretion (average of 6 rats).

treated animals. The rats weighed approximately 250 g. at the beginning of the experiment and lost about 70 g. under thyroid treatment. Thyroid medication began on the sixth day, as indicated by the arrow. The dotted lines in the

figures represent the average behaviour of four untreated control rats, in a simultaneous study.

The administration of thyroid soon leads to a marked increase in the faecal excretion of calcium, which becomes approximately constant at the new level, while the basal metabolic rate (B.M.R.) increases progressively. There is also a decided increase in the urinary excretion of calcium (shown, for clearness, on a larger vertical scale) and the ratio between urinary calcium and faecal calcium, initially very small, does not appear to be greatly altered under thyroid treatment.

Fig. 2 represents another experiment on a series of six rats; thyroid treatment was begun as before on the sixth day, but from the twentieth day (second arrow) the animals also received 20,000 international units of irradiated ergosterol daily by mouth. This dosage has been found in similar experiments on normal rats [Pugsley, 1932] to produce a very marked rise in the urinary calcium excretion with a corresponding decrease in the faecal excretion and without any very pronounced effect on the total calcium balance or the general well-being of the animals. It is clear from the figure that in the present experiments the irradiated ergosterol has again largely diverted the calcium excretion from the faeces to the urine, the total excretion of this element (on practically constant intake) being decreased rather than increased, though still far above the levels observed before thyroid feeding.

Smaller doses of irradiated ergosterol have proved to be less effective. Table I summarises an experiment similar in plan to the last, carried out on another series of six rats. The usual effect of thyroid administration was

Table I. *Effect of administration of 1000 and 10,000 units of irradiated ergosterol daily on the increased calcium excretion induced by thyroid feeding.*

Average of four rats.

Treatment	Day	Urine-Ca mg./day	Faecal Ca mg./day	Ca balance mg./day	Body wt. g.	% increase in O_2 consumption
Control period	2	0·04	4·93	+2·33	224	—
	4	0·06	4·80	+2·76	229	—
	6	0·05	4·59	+2·71	230	0
2 g. desiccated thyroid daily	8	0·04	4·57	+2·63	228	—
	10	0·05	4·60	+1·51	—	—
	12	0·05	5·77	+1·13	221	—
	14	0·03	7·37	−1·40	—	—
	16	0·11	9·25	−3·36	216	—
	18	0·15	10·50	−3·65	—	—
Added 1000 units irradiated ergosterol daily	20	0·11	11·50	−4·61	209	112
	22	0·37	11·50	−3·87	—	—
	24	0·50	12·10	−4·48	—	—
	26	0·57	11·47	−3·79	197	—
	28	0·71	13·67	−5·98	—	—
	30	0·48	12·05	−4·13	188	—
	32	0·74	13·00	−5·34	—	—
10,000 units daily; thyroid continued	34	0·70	13·80	−6·10	180	100
	36	1·22	10·90	−3·72	—	—
	38	1·52	9·17	−2·29	168	—
	40	1·47	9·32	−2·39	—	—
	42	2·47	9·10	−3·17	172	—
	44	2·85	5·58	−0·03	163	—
	46	3·70	4·60	+0·10	—	—
	48	4·24	4·46	−0·30	149	156
	50	3·77	5·62	−0·99	—	—
	52	5·00	3·30	+0·10	—	—
	54	6·72	2·60	−0·92	142	—

observed. The administration of 1000 units of irradiated ergosterol daily, from the twentieth day, may have slightly increased the urinary excretion of calcium but had no discernible effect on the faecal or the total excretions. On the thirty-fourth day the dosage was therefore increased to 10,000 units daily; the table shows that this led in the course of a few days to a definite shift of excretion from faeces to urine, with an unmistakable decline in the total calcium excretion, the negative calcium balance being greatly reduced.

Table II summarises another similar experiment on a series of six rats, in which the dosage selected was 2000 units of irradiated ergosterol daily, from the

Table II. *Effect of administration of 2000 units of irradiated ergosterol daily on the increased calcium excretion induced by thyroid feeding.*

Average of four rats.

Treatment	Day	Urine-Ca mg./day	Faecal Ca mg./day	Ca balance mg./day	Body wt. g.	% increase in O_2 consumption
Control period	2	0·04	4·36	+2·60	231	—
	4	0·05	4·40	+3·17	236	—
	6	0·03	3·77	+3·76	238	0
2 g. desiccated thyroid daily	8	0·06	3·30	+3·64	236	—
	10	0·05	3·00	+3·35	—	—
	12	0·04	4·57	+2·11	231	—
	14	0·02	7·20	−1·04	—	—
	16	0·05	7·30	−1·19	223	—
	18	0·07	8·87	−0·82	—	—
Added 2000 units irradiated ergosterol daily	20	0·02	10·25	−2·21	216	79
	22	0·11	10·12	−1·83	—	—
	24	0·13	9·12	−1·01	—	—
	26	0·22	10·30	−2·12	210	—
	28	0·47	10·48	−2·55	—	—
	30	0·54	10·50	−2·65	207	—
	32	0·48	10·51	−2·75	—	—
	34	0·54	10·77	−2·91	206	83
	36	0·67	10·00	−2·27	—	—
	38	0·38	10·00	−1·98	196	—
	40	0·66	10·00	−2·26	—	—
	42	0·24	10·00	−1·84	188	—
	44	0·40	10·90	−2·90	—	—
	46	0·48	10·80	−2·80	165	—
	48	0·45	11·20	−3·25	—	200

twentieth day. Such doses have been found to reduce the faecal excretion of calcium, in normal animals, very decidedly [Pugsley, 1932]; but in the present experiments, although the faecal excretion and negative balance are somewhat lower and the urinary excretion definitely higher than might have been expected in view of the earlier results, there is not convincing evidence that this dose was in any degree effective.

From a series of 12 rats receiving 2 grains of desiccated thyroid daily, blood was collected by the method previously described [Pugsley, 1932] for determination of the serum-calcium by the method of Clark and Collip [1925]. The values obtained fell within the normal range for rats in this colony, the average being 10·3 mg. per 100 ml. (minimum 10·1, maximum 10·4). This agrees with the general opinion [Thomson and Collip, 1932] that there is no simple relation between thyroid function and the level of the serum-calcium.

The effect of ammonium chloride on calcium excretion was studied in six rats, and the results are summarised in Fig. 3. 1 ml. of a 12·5 % solution of the salt was administered daily by stomach tube from the sixth day; as this had little effect, the dose was increased to 2 ml. from the fourteenth day onward.

A marked diuresis naturally resulted, and the excretion of calcium in the urine was considerably increased, the faecal excretion being scarcely affected. This observation confirms the view of Aub *et al.* [1929] that the thyroid hormone does not affect calcium metabolism merely by producing an acidosis.

Discussion.

The work of Aub *et al.* [1929] on the effect of the thyroid hormone on calcium excretion has, curiously enough, stimulated little experimental study. We have entirely confirmed his view that the thyroid hormone stimulates calcium excretion and promotes loss of calcium from the body, and that the channel of excretion is the intestine rather than the kidney (a matter in which this effect differs strikingly from the increased calcium excretion produced by parathyroid hormone, by ammonium chloride or by very large doses of irradiated ergosterol). It seems at present futile to speculate on the factors responsible for directing calcium excretion to one channel rather than another, but it should be emphasised that in our experiments with thyroid feeding the amount of calcium lost in the faeces was often considerably greater than the total calcium intake. Our work also supports the contention of Aub that the action of the thyroid hormone is not to produce acidosis or parathyroid stimulation, and moreover does not seem to be directly correlated with the increase in basal metabolic rate. In experiments not reported herein, we have found that the injection of parathyroid extract into rats receiving desiccated thyroid increases the total loss of calcium from the body. The failure of Tibbetts *et al.* [1932] to observe a reduction in faecal calcium excretion and negative calcium balance, as we did, after administration of vitamin D, may perhaps be ascribed to inadequate dosage; Taylor and Weld [1932] obtained results similar to ours in dogs.

Summary.

The administration of 2 grains of desiccated thyroid daily, by mouth, to adult rats, leads to a marked increase in calcium excretion and establishment of a negative calcium balance. The increase is observed in the faecal rather than in the urinary calcium. The serum-calcium is not elevated.

The administration of irradiated ergosterol, in doses of 10,000 international units or more daily by mouth, to rats receiving desiccated thyroid reduces the faecal and increases the urinary excretion of calcium, and partly or wholly corrects the negative calcium balance. Smaller doses are comparatively ineffective.

The administration of 250 mg. ammonium chloride daily, by mouth, to normal adult rats leads to a marked increase in the urinary excretion of calcium, with little influence upon the faecal excretion.

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